The *ywaC* promoter is a reporter of cell wall stress

THE YWAC PROMOTER IS A ROBUST REPORTER OF LESIONS IN CELL WALL BIOSYNTHESIS IN *BACILLUS SUBTILIS*

By

Kathryn E. Millar, B.M.Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2006) McMaster University

(Biochemistry and Biomedical Sciences) Hamilton, Ontario

TITLE: The *ywaC* promoter is a robust reporter of lesions in cell wall biosynthesis in *Bacillus subtilis*

AUTHOR: Kathryn E. Millar, B.M.Sc. (McMaster University)

SUPERVISOR: Dr. E.D. Brown

NUMBER OF PAGES: 99

ABSTRACT

The increase in microbes resistant to a wide array of antibiotics has led to the need for the development of novel antimicrobials. However in order to develop new antimicrobials, novel pathways need to be targeted. Teichoic acid is an anionic polymer covalently attached to the cell wall of Gram-positive bacteria. Recent research has demonstrated that teichoic acid genes are indispensable to the viability of *Bacillus subtilis*. This makes teichoic acid biosynthetic proteins ideal candidates for the development of a new antimicrobial. Of the teichoic acid glycerol phosphate (*tag*) genes involved in the biosynthesis of teichoic acid in *B. subtilis* 168, a conditional deletion mutant of *tagD*, whose protein product encodes the proposed glycerol-3-phosphate-cytidylyltransferase, has been previously constructed and was shown to have a lethal phenotype upon depletion of TagD. This was used in a microarray analysis to find genes that were transcriptionally up-regulated upon the depletion of TagD in *B. subtilis* 168. Ten candidate genes were selected from those up-regulated and used in the design of a novel, real-time, cell-based luminescent reporter system that responds to lesions in wall biosynthesis. Characterization of these reporter systems in *tag* gene deletion backgrounds and an examination of their response to antibiotics of various mechanism of action led to the identification of our candidate reporter system P_{ywaC}, a robust reporter of both lesions in teichoic acid and peptidoglycan synthesis. In a proof-of-principle screen, the use of P_{ywaC} as a reporter of lesions in the cell wall was validated. This reporter system is unique in that it combines conventional genetics with a high throughput capacity. It will not only

be amenable for screening small molecules to find inhibitors that impinge on teichoic acid biosynthesis, but it can also be used to probe genetic interactions in *B. subtilis*.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my supervisor Dr. Eric Brown for his support and guidance throughout the course of my project. This has been a very rewarding experience and I have learned a great deal under your direction. I truly appreciate the opportunity to work in your lab. I would also like to extend my gratitude to my supervisory committee members Dr. Ray Truant and Dr. Gerry Wright for their insightful discussions and suggestions throughout my entire career at McMaster. Thank you to the brown lab members for all of your help and for making my time in the lab an enjoyable experience – my experience would not have been the same without you. In particular, Amit Bhavsar and Mike D'Elia stand out for their guidance throughout since the beginning of my work.

To my family members and especially my mother and father, thank you for all of your encouragement throughout my studies. Your continued interest in my education and guidance has always been appreciated and it is because of this unconditional support that I strive to become a better person. Lastly, I would like to thank my boyfriend Rob; whose continuous motivation, encouragement and support has meant a lot to me throughout the years.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PAGE

LIST OF SCHEMES

LIST OF ABBREVIATIONS

DMSO	dimethyl sulfoxide
DOXP	1-deoxy-d-xylulose 5-phosphate
GFP	green fluorescent protein
GlcNAc	N-acetylglucosamine
GroP	glycerol 3-phosphate
LB	luria bertani
ManNAc	N-acetylmannosamine
MIC	minimum inhibitory concentration
MurNAc	N-acetylmuramic acid
POI	promoter of interest
tag	teichoic acid glycerol
tar	teichoic acid ribitol

PREFACE

Sections of this thesis have been submitted for publication under the authorship of Kathryn E. Millar, Amit P. Bhavsar, Ana Tomljenovic, Christoph Schaab, Bernd Hutter and Eric D. Brown. The manuscript was written by Kathryn E. Millar and edited by Amit Bhavsar and Eric Brown. Ana Tomljenovic, a former undergraduate of the Brown lab, mined the TagD dataset and selected ten genes for further characterization. Bernd Hutter and Christoph Schaab from GPC biotech, conducted the TagD microarray and compared the TagD dataset to their previously constructed database containing the transcriptional response of *Bacillus subtilis* to antibiotics of various classes. All other research was conducted by Kathryn E. Millar.

CHAPTER 1 – BACKGROUND INFORMATION

1.1 Antimicrobial Resistance

The middle of the twentieth century marked the beginning of the fight against bacterial pathogens. With the discovery of penicillin in the early 1940's, the "golden age" of antibiotic drug discovery began during which the bulk of currently used antibiotics were discovered (Monaghan and Barrett 2006). The introduction of antibiotics led to the treatment of bacterial diseases that were once considered lethal. However, almost as soon as they were introduced, microbes began acquiring resistance mechanisms to evade the effects of antibiotics. Two years after the introduction of penicillin for therapeutic use, microbes developed resistance mechanisms to this drug, which is a typical time frame for the acquisition of resistance genes (Walsh 2000). The development of resistance to the drugs of the last line defense has emerged in the recent years as can be seen with the rise of vancomycin resistant *Staphylococcus aureus* (Tenover 2006). Thus it is not only a matter of if microbes will acquire resistance mechanisms but when this resistance will arise due to the selection pressure put forth by antibiotics (Livermore 2004).

Microbes can be inherently resistant to an antibiotic class or they can acquire resistance mechanisms through the selective pressure of use of a particular bacterial agent (Tenover 2006). Bacteria may acquire various mechanisms of resistance including genes encoding enzymes that destroy a particular antibiotic before it has a chance to take action as well as developing efflux pumps that will excrete antibiotics from the cell, thereby preventing them from reaching their desired target. Bacteria may also modify an antibiotics intracellular target in such a way that it cannot be recognized (Tenover 2006).

In the years that followed the introduction of penicillin in 1941, a plethora of antibiotics of various classes were developed providing a variety of treatment options to curtail the spread of bacterial infections (Powers 2004). However in the past thirty years, only two novel classes of antibiotics have been introduced with the majority of new antimicrobial agents being chemically modified forms of previously discovered antibacterial classes (Figure 1.1) (Powers 2004). Furthermore, the current antibiotics in use target a limited repertoire of pathways in the bacterial cell namely interference with cell wall synthesis, DNA synthesis, protein synthesis and inhibition of a metabolic pathway (Tenover 2006). Due to the widespread use of antibiotics and with conventional antibiotics targeting a limited number of pathways in the bacterial cell, identifying novel antibacterial targets may be an effective strategy for developing new antimicrobials, and one that may be subject to less inherent resistance.

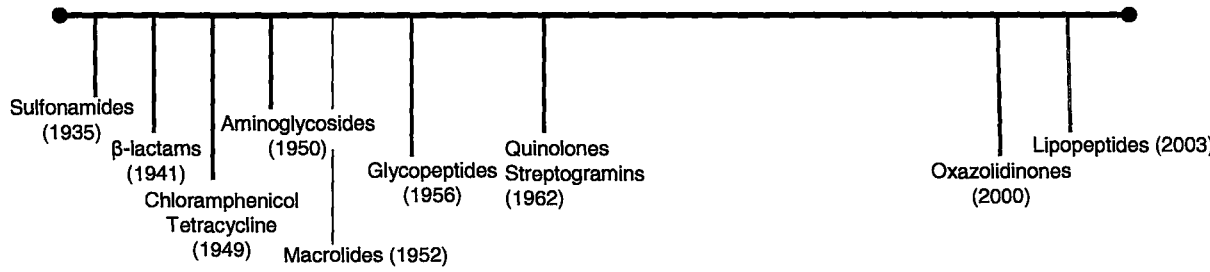


Figure 1.1 Timeline of antibacterial agents introduced for therapeutic use. The majority of antibacterial agents were introduced prior to 1962 with only three antibacterial agents approved for therapeutic use since that time.

1.2 The Gram-positive cell wall

The bacterial cell wall is an essential structure that provides protection to the protoplast, withstands turgor pressure and maintains cell shape (Archibald 1993). The three main components of the cell wall include proteins, peptidoglycan and anionic polymers, with the majority of the cell wall being composed of the latter two components found in equal proportions (Bhavsar and Brown 2006). While both peptidoglycan and anionic polymers constitute a large portion of the cell wall, the majority of current antimicrobials exert their mechanism of action against the peptidoglycan biosynthetic pathway.

1.2.1 Teichoic Acid Biosynthesis

Wall teichoic acid is an anionic polymer covalently attached to peptidoglycan in Gram-positive bacteria. Teichoic acid is found among many Gram-positive species including *Staphylococcus aureus,* a Gram-positive pathogen associated with many hospital and community acquired infections. This phosphate-rich polymer constitutes a major portion of the cell wall however its function in the cell remains unknown. The

predominant teichoic acid polymer produced by *Bacillus subtilis* 168 is 1, 3-linked poly (glycerol phosphate) and the bulk of the genes involved in its biosynthesis are known as the teichoic acid glycerol (*tag*) genes which are organized into two divergently transcribed operons (Figure 1.2). Three other *tag* genes, *tag*O, *tag*G, and *tag*H, located outside of this region are also involved in teichoic acid biosynthesis. There are four reactions involved in the biosynthesis of teichoic acid; linkage unit synthesis, main chain synthesis, polyol phosphate activation and glucosylation, and export and attachment to peptidoglycan. While the main chain polymer of teichoic acid varies among Gram-positive organisms, the linkage unit is widely conserved. Scheme 1 provides a diagrammatic explanation of teichoic acid synthesis.

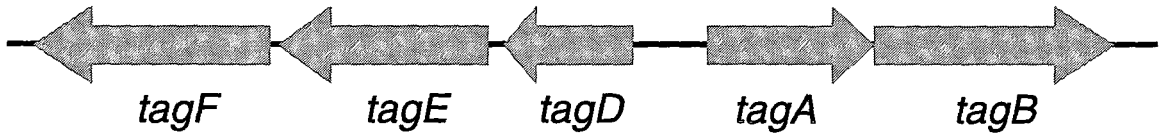
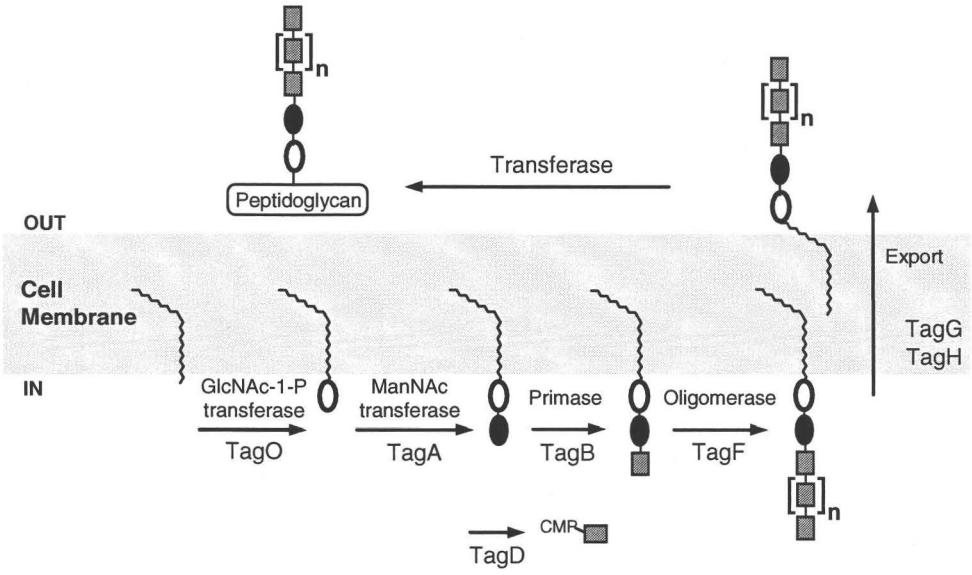


Figure 1.2. The divergent *tag* operon in *B. subtilis 168*. The *tag* genes code for poly(glycerol phosphate) synthesis. Three other *tag* genes (*tagO, tagGH*), have been found to be located outside of this region.



Scheme 1. Teichoic acid assembly and export. The teichoic acid polymer is synthesized intracellularly on a membrane-embedded carrier and is exported out of the cell and attached to the 6-hydroxyl of MurNAc of peptidoglycan.

The linkage unit disaccharide of wall teichoic acid contains N-acetylmannosamine-β(1-4)-N-acetylglucosamine and is connected to peptidoglycan through a phosphodiester bond to carbon 6 of N-acetylmuramic acid (MurNAc) (Figure 1.3). The synthesis of teichoic acid occurs on a membrane-embedded carrier undecaprenol-phosphate, a shared building block between peptidoglycan and teichoic acid biosynthesis (Anderson, Hussey et al. 1972). N-acetyl-glucosamine-1-phosphate (GlcNAc-1-P) is thought to be transferred onto undecaprenol-phosphate through the action of TagO, followed by the addition of N-acetyl-mannosamine (ManNAc) by TagA.

Main chain synthesis of teichoic acid occurs through the incorporation of an activated form of glycerol phosphate (GroP), which is CDP-glycerol in the case of *B.*

subtilis 168, onto the linkage unit to produce a polymer of glycerol phosphate. TagD is the CDP-glycerol pyrophosphorylase that synthesizes this activated monomeric unit and is added to the growing polymer through the action of TagB and TagF (Park, Sweitzer et al. 1993). TagB, the Tag primase (Bhavsar, Truant et al. 2005), adds one GroP residue to the 4-hydroxyl of ManNAc to which the successive addition of multiple GroP residues are added by the Tag polymerase, TagF (Schertzer and Brown 2003). This repeating main chain polymer can then be decorated with glucose substituents through the action of TagE and exported out of the cell by TagGH (Bhavsar and Brown 2006). The dispensability of *tag* genes has been investigated to determine if the teichoic acid biosynthetic pathway is a plausible therapeutic target.

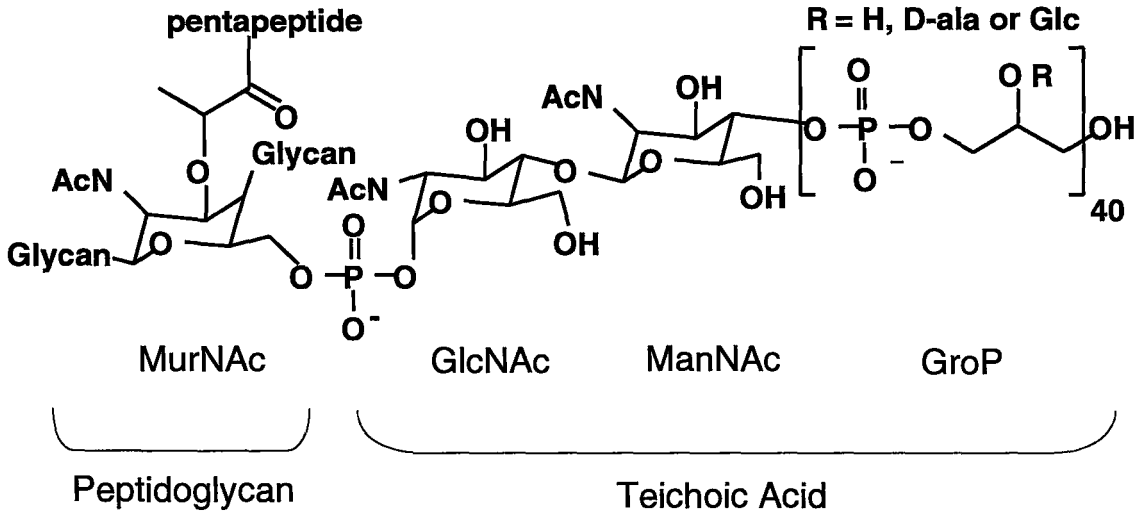


Figure 1.3. Structure of poly(glycerol phosphate) teichoic acid in *Bacillus subtilis* 168. The teichoic acid polymer is attached to peptidoglycan through a phosphodiester bond between the 6-hydroxyl of MurNAc and the linkage unit. The repeating polyol in *B. subtilis* 168 consists of glycerol-3-phosphate.

1.3. Teichoic Acid Dispensability Studies

Temperature sensitive mutants created through chemical mutagenesis, were among the first evidence that the *tag* genes involved in the biosynthesis of teichoic acid, in particular *tagB, tagD,* and *tagF*, are indispensable to *B. subtilis* 168 (Brandt and Karamata 1987; Briehl 1989; Pooley, Abellan et al. 1991). A xylose-based conditional complementation system was recently employed to confirm the essentiality of *tagB, tagD,* and *tagF* to *B. subtilis* 168 in a defined genetic background and at a physiologically relevant temperature (Bhavsar, Beveridge et al. 2001; Bhavsar, Erdman et al. 2004). This complementation system allowed for the precise deletion of *tagB, tagD,* and *tagF* at the *tag* locus in the presence of an ectopically integrated xylose inducible complementing copy of the gene at the *amyE* locus (Bhavsar, Beveridge et al. 2001; Bhavsar, Erdman et al. 2004). The controlled depletion of the *tag* gene products showed a lethal phenotype that was characterized by a rod to sphere morphology transition (Bhavsar, Beveridge et al. 2001; Bhavsar, Erdman et al. 2004).

While it has long been thought that the essentiality of teichoic acid to *B. subtilis* extends to other Gram-positive organisms, it was found that this is not the case for *S. aureus,* which encodes a poly (ribitol phosphate) teichoic acid polymer. It has been recently shown that the deletion of *tarO*, the proposed GlcNAc-1-P transferase is dispensable in *S. aureus* (Weidenmaier, Kokai-Kun et al. 2004), however the remainder of the later-acting teichoic acid ribitol (*tar*) genes (*tarB, tarD, tarF, tarIJ* and *tarH*), remain indispensable (D'Elia, Pereira et al. 2006). Surprisingly, these late-acting *tar*

genes become dispensable in the *tarO* mutant background (D'Elia, Pereira et al. 2006). Although *tarO* is dispensable in *S. aureus,* it remains a virulence target and thus teichoic acid is still a viable therapeutic target as the absence of teichoic acid in *S. aureus* has been shown to prevent colonization in mice (Weidenmaier, Kokai-Kun et al. 2004).

1.4. Screening methodology

Cell-based screening was the method used in the past to identify the bulk of currently used antibiotics. While this type of screening method has proven successful in the past, it is not without its limitations. Overtime and with the advent of high throughput screening in the 1990s, a shift has resulted in the way screening for novel antimicrobials is being conducted. There has been a move towards target-based biochemical screening, a method that uses a one-target-one-compound concept for identifying inhibitors. New techniques are emerging that build upon both target-based and cell-based screening and can be designed to find inhibitors of currently unexploited pathways in the bacterial cell.

1.4.1 Target-based biochemical screening

Target-based biochemical screening aims to identify compounds with established mechanisms of action. The desired protein target is previously determined and compounds are added to find small molecules that interact with this protein (Spring 2005). In order for a protein to be considered a potential target for a novel antimicrobial, it must be essential to cell survival and found to be conserved among an array of bacteria (Pucci 2006). In addition to these two criterions, the inhibitor would also ideally be

bactericidal as well as having a limited toxicity in humans (Pucci 2006). While target-based screening studies can be successful in identifying active compounds against their bacterial protein target, this type of screening is limited in its ability to find bio-active compounds. Compounds may not have established antimicrobial properties and may not be able to access their target due to issues with cell permeability and being a possible substrate for efflux (Monaghan and Barrett 2006). Despite the fact that this screening method may be deemed problematic due to penetration issues with respect to lead compounds, this screening technique is valuable in that it can select for poorly penetrating compounds that may be suitable for chemical optimization (Rosamond and Allsop 2000).

1.4.2 Cell death screens

Cell-based screening differs from target-based biochemical screening in that it aims to identify a phenotype of irregular cell morphology when small molecules are added to normal bacterial cells (Spring 2005). This screening approach is advantageous as it selects for compounds that penetrate bacterial cells although it fails to elucidate the mechanism of action of an unknown compound as the target is not known. Cell-based screening can identify penetrable compounds, however many compounds identified are toxic to humans and thus are not ideal compounds for optimization (Fischer, Brunner et al. 2004).

1.4.3 Promoter-reporter screening systems

The aforementioned screening methods are each unique with their own sets of advantages and disadvantages. Designing a screening method that could implement the

positive aspects of each screening technique would prove to be a successful method and one that could identify novel antimicrobial agents that target previously unexploited pathways at an accelerated pace. Promoter-reporter screening systems provide an attractive alternative to cell and target-based biochemical screening as it would select for bio-active compounds with the ability to identify the pathway compounds are targeting.

There are various reporters available for the development of promoter-reporter assays. Two main reporter systems used to study gene expression are green fluorescent protein (GFP) and luminescence. GFP emits green fluorescence when exposed to light at a wavelength of approximately 500 nm. While it is possible to use GFP as a reporter, it is problematic as it is not very sensitive due to a high level of background fluorescence and it does not have the ability to monitor gene expression in real-time (Qazi, Counil et al. 2001). Luminescence is a sensitive measure that will only detect live, viable cells (Francis, Joh et al. 2000). It is a unique system in that it can be designed to be noninvasive and can be used to monitor continuous cell growth. By designing a reporter system that uses all of the *lux* genes necessary for luminescence, continuous promoter activity can be monitored without the need to add in exogenous substrate.

Two separate studies have taken advantage of promoter-reporter screening using luminescence as the reporter by designing fatty acid pathway specific reporters using genes that respond to inhibitors of fatty acid biosynthesis (Fischer, Brunner et al. 2004; Hutter, Fischer et al. 2004). In addition to designing a fatty acid pathway specific

reporter, Hutter, et. al., designed reporters specific to DNA, protein and cell wall biosynthetic pathways using genes that were transcriptionally activated in response to compounds targeting these pathways. In each study, the transcriptional response of *B. subtilis* 168 to well characterized antibiotics was observed to identify stress-inducible promoters. These promoters were cloned into a reporter plasmid upstream of *luxAB*, the genes that encode luciferase. In order to visualize luminescence from the reporter systems, the addition of luciferin, the exogenous substrate for luciferase was required.

The studies mentioned above were designed to target pathways to which current antimicrobials already target. While there are still viable targets within these pathways, implementing a reporter system with the ability to respond to lesions in an unexplored pathway would be ideal for drug discovery research as it might lead to the identification of novel chemical entities with unique mechanisms of action.

1.5 Research Objectives

The goal of this research project was to design a promoter-reporter system that would respond to lesions in teichoic acid biosynthesis, which could then be implemented in a small molecule screen to find inhibitors of this previously unexploited pathway.

To this end, the *tagD* conditional mutant was used to perform RNA transcriptional analysis of TagD-depleted cells. Genes found to be transcriptionally activated in response to TagD-depletion were of interest, however genes that were transcriptionally activated by antibiotics that target cell wall synthesis were also studied. From this microarray analysis, the promoter regions of transcriptionally activated genes were used to develop a novel, real-time reporter system. After testing the candidate reporters in various teichoic acid mutant backgrounds, the promoter region of *ywaC* emerged as the most robust reporter of lesions in both peptidoglycan and teichoic acid synthesis. A proof-of-principle screen has validated the ability of the P_{ywaC} reporter system to respond to lesions in teichoic acid and peptidoglycan biosynthesis.

CHAPTER 2 – EXPERIMENTAL PROCEDURES

2.1 Bacterial Strains, Reagents, and General Methods.

Tables 2.1 and 2.2 list the strains, plasmids and primers used in this study. All cloning was performed in *E. coli* cloning strain Novablue (Novagen, Madison, WI.) according to previously described protocols (Sambrook 1989). Transformations in *B. subtilis* were conducted according to established protocols (Cutting 1994). *E. coli* and *B. subtilis* were grown at 37 °C and 30 °C respectively in Luria-Bertani (LB) liquid medium with aeration at 250 rpm or on solid LB agar supplemented with the appropriate antibiotics. Antibiotic selection was as follows: 50 μg/ml ampicillin, 12.5 μg/ml lincomycin, 0.5 μg/ml erythromycin, 5 μg/ml chloramphenicol and 200 μg/ml spectinomycin. When required, xylose was used at a final concentration of 2 % (wt/vol). Molecular cloning reagents were obtained from New England Biolabs (Beverly, MA). All chemicals were purchased from Sigma (Oakville, ON).

Table 2.1. Bacterial strains and plasmids used in this study

Strain or Plasmid	Description	Reference
E. coli		
Novablue	*endA1 hsdR17(rk12-mk12+)supE44 thi-1 recA1 gyrA96 relA1 lac [F' pro A+ B+ lacIqZ delta M15::Tn10] TcR*	Novagen
MC1061	*[araD139], Δ(araA-leu)7679, Δ(codB-lacI)3, galK16, galE15, lambda-, mcrA-, e14-, relA1, rpsL150(strR), spoT1, mcrB-, hsdR2*	CGSC
B. subtilis		
EB6	*hisA1 argC4 met C3*	(Briehl 1989)
EB240	*hisA1, argC4, metC3, amyE::xylR* P_{xylA} *tagD cat86 tagD*::Spec^r	(Bhavsar, Beveridge et al. 2001)
EB323	*hisA1, argC4, metC3, amyE::xylR* P_{xylA} *yacN cat86 yacN*::Spec^r	(Campbell and Brown 2002)
EB633	*hisA1, argC4, metC3, amyE::xylR* P_{xylA} *tagB cat86 tagB*::Spec^r	(Bhavsar, Erdman et al. 2004)
EB669	*hisA1, argC4, metC3, amyE::xylR* P_{xylA} *tagF cat86 tagF*::Spec^r	(Bhavsar, Erdman et al. 2004)
EB1385	EB6 with P_{ywaC} *luxABCDE,* erm^R*, bla*	This Study
EB1399	EB240 with P_{yhaU} *luxABCDE,* erm^R*, bla*	This Study
EB1400	EB240 with P_{yktB} *luxABCDE,* erm^R*, bla*	This Study
EB1401	EB240 with P_{yqiW} *luxABCDE,* erm^R*, bla*	This Study
EB1402	EB240 with P_{ywaC} *luxABCDE,* erm^R*, bla*	This Study
EB1403	EB240 with P_{yvfT} *luxABCDE,* erm^R*, bla*	This Study
EB1404	EB240 with P_{yfiB} *luxABCDE,* erm^R*, bla*	This Study
EB1405	EB240 with P_{yxeI} *luxABCDE,* erm^R*, bla*	This Study
EB1406	EB240 with P_{murB} *luxABCDE,* erm^R*, bla*	This Study
EB1407	EB240 with P_{yubB} *luxABCDE,* erm^R*, bla*	This Study
EB1408	EB240 with P_{ydbO} *luxABCDE,* erm^R*, bla*	This Study
EB1432	EB669 with P_{yubB} *luxABCDE,* erm^R*, bla*	This study
EB1433	EB669 with P_{yktB} *luxABCDE,* erm^R*, bla*	This study
EB1434	EB669 with P_{murB} *luxABCDE,* erm^R*, bla*	This study
EB1485	EB633 with P_{yubB} *luxABCDE,* erm^R*, bla*	This study
EB1493	EB6 with P_{serS} *luxABCDE,* erm^R*, bla*	This Study
EB1546	EB669 with P_{ywaC} *luxABCDE,* erm^R*, bla*	This study
EB1547	EB669 with P_{yvfT} *luxABCDE,* erm^R*, bla*	This study
EB1548	EB669 with P_{yhaU} *luxABCDE,* erm^R*, bla*	This study
EB1550	EB633 with P_{yktB} *luxABCDE,* erm^R*, bla*	This study
EB1551	EB633 with P_{ywaC} *luxABCDE,* erm^R*, bla*	This study
EB1552	EB633 with P_{murB} *luxABCDE,* erm^R*, bla*	This study
EB1553	EB633 with P_{yhaU} *luxABCDE,* erm^R*, bla*	This study
EB1555	EB633 with P_{yvfT} *luxABCDE,* erm^R*, bla*	This study
EB1593	EB323 with P_{ywaC} *luxABCDE,* erm^R*, bla*	This study
EB1594	EB323 with P_{serS} *luxABCDE,* erm^R*, bla*	This study

Plasmids

pMUTIN4	Integrational vector for *B. subtilis*	(Vagner, Dervyn et al. 1998)
pBluescript SK II+	General cloning vector	Stratagene
pBS_P$_{murB}$	pBluescript containing promoter region of *murB*	This Study
pBS_P$_{serS}$	pBluescript containing promoter region of *serS*	This Study
pBS_P$_{ydbO}$	pBluescript containing promoter region of *ydbO*	This Study
pBS_P$_{yfiB}$	pBluescript containing promoter region of *yfiB*	This Study
pBS_P$_{yhaU}$	pBluescript containing promoter region of *yhaU*	This Study
pBS_P$_{yktB}$	pBluescript containing promoter region of *yktB*	This Study
pBS_P$_{yqiW}$	pBluescript containing promoter region of *yqiW*	This Study
pBS_P$_{yubB}$	pBluescript containing promoter region of *yubB*	This Study
pBS_P$_{yvfT}$	pBluescript containing promoter region of *yvfT*	This Study
pBS_P$_{yxeI}$	pBluescript containing promoter region of *yxeI*	This Study
pBS_P$_{ywaC}$	pBluescript containing promoter region of *ywaC*	This Study
pSB2025	*luxABCDE* inserted into pSL1190	(Qazi, Counil et al. 2001)
pLuxErm	pSB2025 derivative containing erythromycin resistance cassette	This study
pLuxErmP$_{murB}$	pLuxErm derivative containing promoter region of *murB*	This study
pLuxErmP$_{serS}$	pLuxErm derivative containing promoter region of *serS*	This study
pLuxErmP$_{ydbO}$	pLuxErm derivative containing promoter region of *ydbO*	This study
pLuxErmP$_{yfiB}$	pLuxErm derivative containing promoter region of *yfiB*	This study
pLuxErmP$_{yhaU}$	pLuxErm derivative containing promoter region of *yhaU*	This study
pLuxErmP$_{yktB}$	pLuxErm derivative containing promoter region of *yktB*	This study
pLuxErmP$_{yqiW}$	pLuxErm derivative containing promoter region of *yqiW*	This study
pLuxErmP$_{yubB}$	pLuxErm derivative containing promoter region of *yubB*	This study
pLuxErmP$_{yvfT}$	pLuxErm derivative containing promoter region of *yvfT*	This study
pLuxErmP$_{yxeI}$	pLuxErm derivative containing promoter region of *yxeI*	This study
pLuxErmP$_{ywaC}$	pLuxErm derivative containing promoter region of *ywaC*	This study

Table 2.2 Sequences of the oligonucleotide primers used in this study

Oligonucleotide	Sequence[a]	Restriction Site
Erm-F	5'-cgcctgcagttaagagtgtgttgatagtgc-3'	*Pst*I
Erm-R	5'-cgcgcgctcgaggcgactcatagaattatttcc-3'	*Xho*I
P_{murB}-F	5'-cgcgaattcaaacagcctgccgggcatcacg-3'	*Eco*RI
P_{murB}-R	5'-cgcgtcgacatttgcttttctatttttttaa-3'	*Sal*I
P_{sers}-F	5'-cgcccatggcgtatcaattcatggagccg-3'	*Nco*I
P_{sers}-R	5'-cgcgtcgacgagccctcatccccataaaggg-3'	*Sal*I
P_{ydbO}-F	5'-cgcgaattcatatcgtcacaaacgctgcgc-3'	*Eco*RI
P_{ydbO}-R	5'-ccgcccatggattctattacatagtatggttc-3'	*Nco*I
P_{yfiB}-F	5'cgcgaattcacactgacagcgattgtcgcc 3'	*Eco*RI
P_{yfiB}-R	5'ccgcccatggaaacccctttttactcgattg 3'	*Nco*I
P_{yhaU}-F	5'-cgcgaattcgcaggaaacacaatcggcaaaaacg-3'	*Eco*RI
P_{yhaU}-R	5'-cgcgtcgacagaaagaaaatcgtgaatcagtttc-3'	*Sal*I
P_{yktB}-F	5'-ccgcccatggaaatgtttcttgaattcgatc-3'	*Nco*I
P_{yktB}-R	5'-cgcgtcgacatgctaagtatacatattttatc-3'	*Sal*I
P_{yqiW}-F	5'-cgcgaattccacaagcagcataacggcttc-3'	*Eco*RI
P_{yqiW}-R	5'-cgcccatggttttcatcatctgttcttatg-3'	*Nco*I
P_{yubB}-F	5'-cgcgaattccggtgttgggaattttatcgg-3'	*Eco*RI
P_{yubB}-R	5'-cgcgtcgacattagacatttatttttttac-3'	*Sal*I
P_{yvfT}-F	5'-ccgcccatggaataccaacgtccctgatc-3'	*Nco*I
P_{yvfT}-R	5'-cgcgtcgactgtttgacccctgattctt-3'	*Sal*I
P_{ywaC}-F	5'-cgcgaattccttgcctcatacgggattgcc-3'	*Eco*RI
P_{ywaC}-R	5'-cgcgtcgacttaacggaactttatccgctg-3'	*Sal*I
P_{yxeI}-F	5'-cgcgaattcttcaaaatacgcatacaaatg-3'	*Eco*RI
P_{yxeI}-R	5'-cgcgtcgactttcaattcagattaatatc-3'	*Sal*I
luxA-R	5'-gccacctctgctatacgccgcgg-3'	
P_{murB}ver[b]	5'-gagcgggagaatataccgtttcg-3'	
P_{sers}ver	5'-gcgtatgcaagaacgcggctgg-3'	
P_{ydbO}ver	5'-cctgcgatttatcttggtgtggc-3'	
P_{yfiB}ver	5'ggcccggctgtagcggaaggcggg 3'	
P_{yhaU}ver	5'-ggtcaatagatactgaaaaatgc-3'	
P_{yktB}ver	5'-gccagcttcgtttcattcatataagc-3'	
P_{yqiW}ver	5'-gggtgtatgtgtataaacttctcc-3'	
P_{yubB}ver	5'-cgggctgatcacgatcacgatcg-3'	
P_{yvfT}ver	5'-cagacaaagtgttggcgctgatc-3'	
P_{ywaC}ver	5'-cgtcctcatacgttaaccgcatcg-3'	
P_{yxeI}ver	5'-cgacatgaaggagatgcaggatg-3'	

[a] Restriction enzyme sites are underlined

[b] Primers verifying pLuxErmPOI integrated into various *B. subtilis* strains

2.2 Culturing of a *B. subtilis tagD* conditional mutant.

A *B. subtilis tagD* deletion mutant with a conditionally expressed copy of *tagD* under the control of the xylose promoter (Bhavsar, Beveridge et al. 2001) was grown overnight in LB supplemented with chloramphenicol and xylose. Working cultures were inoculated at an OD_{600} of 0.05 in 20 ml LB supplemented with xylose and cultures were incubated at 37 °C and 200 rpm. After the culture reached an OD_{600} of 0.5, the cell suspension was spun gently and the cell pellet was resuspended at an OD_{600} of 0.05 in 100 ml of fresh medium without xylose. The culture was divided into halves and xylose was added to one half of the culture. Bacterial cultures were incubated as described above. Cultures were diluted to an OD_{600} of 0.05 in fresh medium when the culture grown in the absence of xylose reached an OD_{600} of 0.5 (one cycle of growth).

The cultures grown in the absence of xylose were growth deficient compared to the cultures grown in the presence of xylose. This growth deficiency was already well pronounced in the first two cycles of growth without xylose. In the third growth cycle, the cultures grown in the absence of xylose did not increase in optical density.

2.3 Gene expression profiling of the tagD conditional mutant.

All cultures were grown in triplicate as indicated above. Experimental details, RNA preparation and hybridization, were identical to the conditions described above and in (Hutter, Schaab et al. 2004). The time points used for RNA preparation were (a) after end of growth cycle 1, (b) after end of growth cycle 2 and (c) 60 minutes after entering into growth cycle 3.

2.4 Microarray Data Analysis.

The transcriptional response of *B. subtilis* genes was analyzed in order to identify genes that were specifically up-regulated in response to TagD depletion. The following set of criteria was used to determine which genes were transcriptionally activated; (i) The gene was up-regulated at least 2-fold with respect to the corresponding control sample (EB240 plus xylose) and (ii) Up-regulation was significant at a 5% significance level (t-test). This set of criteria determined that 180 genes were transcriptionally activated in response to TagD depletion. To narrow down the list of 180 up-regulated genes to ten that would then be used to construct reporter plasmids, an expression profile database containing the transcriptional response of *B. subtilis* 168 to 37 well-characterized antimicrobials was used for comparison (Hutter, Schaab et al. 2004). Two categories of transcriptional response were identified; (i) genes whose transcription was increased in response to TagD depletion and not in response to the 37 antimicrobials, and (ii) genes whose transcription was increased in response to TagD depletion and to cell wall active antibiotics. Genes were also short-listed on the basis of conservation within other Gram-positive species as well as being up-regulated at least 4-fold with respect to the corresponding control sample in the first round of TagD- depletion. Five of the 10 selected genes were specific to TagD depletion and the remaining 5 responded to cell wall active antibiotics.

2.5 Construction of Reporter Plasmids

An erythromycin resistance cassette amplified from pMUTIN4 using primers 5'-cgcctgcagttaagagtgtgttgatagtgc-3' and 5'-cgcgcgctcgaggcgactcatagaattatttcc-3', digested with *Xho*I and *Pst*I restriction enzymes was inserted into pSB2025 digested with the same sites to generate pLuxErm (Figure 2.1).

To clone candidate promoters into pLuxErm, 750 base pairs upstream of the putative translational start site of the gene of interest was amplified from *B. subtilis* 168 genomic DNA using the primers listed in Table 2.2. This polymerase chain reaction (PCR) product was cloned into pBluescript digested with *EcoRV* and subsequently ligated into pLuxErm in the multiple cloning site upstream of *luxA* using a combination of the following restriction enzymes: *Eco*RI, *Nco*I and *Sal*I, yielding pLuxErm containing the promoter of interest (POI).

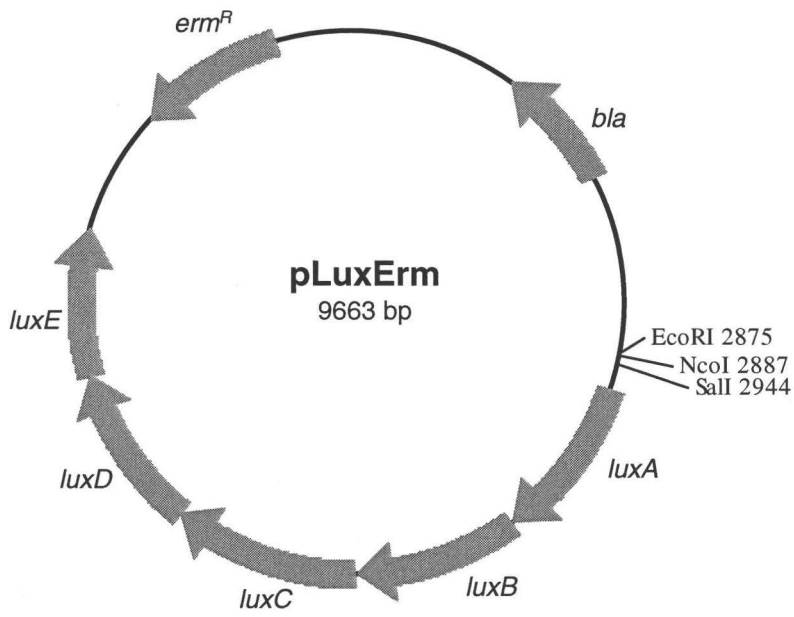


Figure 2.1. Plasmid map of pLuxErm. pLuxErm is a derivative of pSB2025 (Qazi, Counil et al. 2001) which contains the lux operon. pLuxErm contains an origin of replication for *E. coli* but not *B. subtilis*. The erythromycin resistance cassette allows for selection in *B. subtilis*. Test promoters were cloned into pLuxErm through the multiple cloning site upstream of *luxA*.

2.6 Generation of Reporter Strains

The reporter plasmids pLuxErmPOI were transformed into the following *B. subtilis* strains after passage through *E. coli* MC1061to concatamerize the DNA: EB6, EB240, EB633 and EB669 and selected on LB supplemented with spectinomycin, lincomycin, erythromycin and xylose for EB240, EB633 and EB669 and erythromycin was used for selection in EB6. To verify that a single integration event had occurred, genomic DNA was prepared from spectinomycin, lincomycin and erythromycin resistant EB240, EB633 and EB669 transformants and lincomycin and erythromycin resistant EB6 transformants and analyzed by PCR using a reverse primer that annealed to *luxA* and a forward primer that annealed upstream of the amplified promoter region of the gene of interest (Table 2.2).

2.7 Reporter Assay

2.7.1 TagB-, TagD-, TagF-, YacN- depletion studies

One milliliter of an overnight culture of EB240, 323, 633 and 669 transformants, grown in LB supplemented with spectinomycin, erythromycin and xylose was pelleted and resuspended into 1ml of fresh LB containing spectinomycin and erythromycin with or without xylose. An initial 100-fold dilution was made into LB supplemented with erythromycin and spectinomycin or LB supplemented with erythromycin, spectinomycin and xylose in white, clear-bottom 96-well plates (Perkin Elmer, Woodbridge, ON). A second 100-fold dilution was made as required if growth in the absence of xylose approached an OD_{600} ~0.2 after four hours of growth. Growth and luminescence were

monitored for a period of twelve hours using the Envision (Luminescence plate reader) (Perkin Elmer, Woodbridge, ON). All experimental conditions were run in triplicate.

2.7.2 Antibiotic Studies

The minimum inhibitory concentration (MIC) of each antibiotic was determined in white, clear-bottom 96-well plates (Perkin Elmer, Woodbridge, ON). Concentrations two-fold and four-fold above and below the MIC as well as the MIC concentration of each antibiotic was tested. A 100-fold dilution of EB1385 was made into 200μL of LB supplemented with erythromycin from an overnight culture grown in the same medium. Cells were grown in white, clear-bottom 96-well plates (Perkin Elmer, Woodbridge, ON). As cells approached an OD_{600} ~0.2, antibiotics were added to the cells at the following concentrations (in μg/ml); 0.5, 1, 2, 4, 8 ampicillin, fosmidomycin, cefotaxime and trimethoprim; 1, 2, 4, 8, 16 nalidixic acid and tetracycline; 128, 256, 512, 1024, 2048 cycloserine and fosfomycin; 64, 128, 256, 512, 1024 bacitracin; 0.25, 0.5, 1, 2, 4, novobiocin and triclosan; 0.125, 0.25, 0.5, 1, 2 ramoplanin and vancomycin; and 2, 4, 8, 16, 32, tunicamycin and neomycin. An additional concentration of tunicamycin was tested against the P_{ywaC} reporter strain at 1 μg/ml. Growth and luminescence were monitored for a period of twelve hours using the Envision (luminescence plate reader). All experimental conditions were run in triplicate.

2.8 High throughput screening

2.8.1 Prestwick Screen

High throughput screening was done using the Prestwick Library (Prestwick Chemical Inc., Washington, DC), which contains 1120 compounds. Bacitracin (1 μM) was used as the high control and dimethyl sulfoxide (DMSO) was used as the low control. The screen was conducted essentially the same way as described above for the antibiotic and Tag protein depletion studies. The P_{ywaC} reporter strain (EB1385) was grown to an OD_{600} ~0.2, after which time, compounds were added using the Biomek FX liquid handler (Beckman Coulter Inc., Fullerton, California) to a final concentration of 10μM. Growth and luminescence were monitored for a period of twelve hours using the Envision (Perkin Elmer, Woodbridge, ON). All experimental conditions were run in duplicate. It should be noted that at the time of writing, no MAC ID numbers were available for compounds in the Prestwick library and thus the compound's trivial names were used. These names are searchable in the Prestwick Database.

2.8.2 Z' Factor

The Z'-factor was determined using Bacitracin (1 μM) as the high control and DMSO as the low control. The experiment was run essentially the same way as described above however after the P_{ywaC} reporter strain (EB1385) was grown to an OD_{600} ~0.2, the Biomek FX liquid handler (Beckman Coulter Inc., Fullerton, California) added the high control to half of the wells in clear-bottom, 96-well plates (Perkin Elmer, Woodbridge, ON) and the low control to the other half of the wells. Five clear-bottom 96-well plates were used to conduct the experiment.

CHAPTER 3 – RESULTS

3.1 The search for genes transcriptionally activated by TagD depletion

RNA transcriptional analysis of TagD-depleted cells was conducted in *B. subtilis* 168 to find genes that were transcriptionally activated in response to this depletion. The TagD conditional mutant was used under both replete (2 % xylose) and deplete (0 % xylose) conditions and the results were compared (Figure 3.1). One hundred and eighty genes were identified to be transcriptionally activated in response to TagD depletion. The transcriptional profiles of these genes were compared to data sets of genes transcriptionally activated by known cell wall antibiotics (Hutter, Schaab et al. 2004) and were classified according to up-regulation under TagD-depletion exclusively, or those that were also up-regulated in response to cell wall active antibiotics. In addition, genes were also classified according to their conservation among Gram-positive bacteria. From this data analysis, 10 candidate genes were identified, 5 of which were relatively specific to TagD depletion and the remaining 5 of which were up-regulated in response to lesions in teichoic acid and peptidoglycan biosynthesis (Table 3.1).

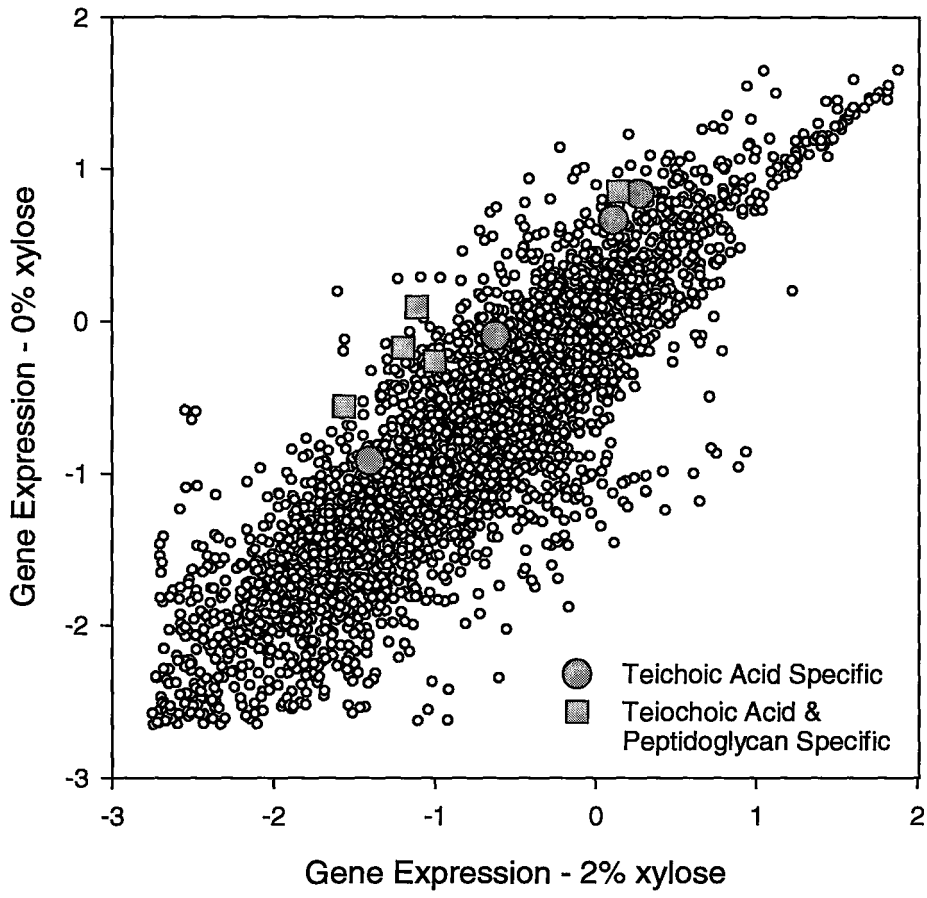


Figure 3.1. Microarray transcriptional analysis of the *tagD* conditionally complemented mutant. The transcriptional response of every *B. subtilis* 168 open reading frame is represented as a function of expression in the presence or absence of xylose in strain EB240. The ten genes selected for further characterization were categorized into genes activated in response to lesions in teichoic acid biosynthesis (●) and genes activated in response to lesions in teichoic acid and peptidoglycan synthesis (■).

Table 3.1 Genes from the *B. subtilis* TagD-depletion microarray analysis short-listed based on their transcriptional up-regulation to TagD-depletion and response to cell wall biosynthesis inhibitors[a]

Gene	Accession Number	Function	Specificity[b]
yktB	BG11817	unknown; similar to unknown proteins	teichoic acid
yqiW	BG11726	unknown; similar to unknown proteins from B. subtilis	teichoic acid
yubB	BG13951	unknown; similar to bacitracin resistance protein (undecaprenol kinase)	teichoic acid
yvfT	BG12445	unknown; similar to two-component sensor histidine kinase [YvfU]	teichoic acid
yxeI	BG11885	unknown; similar to penicillin amidase	teichoic acid
murB	BG10228	UDP-N-acetylenolpyruvoylglucosamine reductase	teichoic acid & peptidoglycan
ydbO	BG12082	unknown; similar to cation efflux system	teichoic acid & peptidoglycan
yhaU	BG12997	unknown; similar to Na+/H+ antiporter	teichoic acid & peptidoglycan
yfiB	BG11849	unknown; similar to ABC transporter (ATP-binding protein)	teichoic acid & peptidoglycan
ywaC	BG10553	unknown; similar to GTP-pyrophosphokinase	teichoic acid & peptidoglycan

[a] Response to cell wall biosynthesis inhibitor determined by comparison to database containing transcriptional response of *B. subtilis* 168 to antibiotics of various classes (Hutter, Schaab et al. 2004).
b Criteria for specificity designation as teichoic acid was determined from genes whose transcription was increased in response to TagD-depletion and showed a limited response to the 37 different antimicrobials (Hutter, Schaab et al. 2004). Teichoic acid and peptidoglycan specificity was determined from genes whose transcription was increased in response to TagD-depletion as well as to cell wall active compounds.

3.2 Generating reporter strains specific to teichoic acid and peptidoglycan biosynthesis

A reporter system using the luminescence (*lux*) genes from *Photorhabdus luminescens* was constructed. *P. luminescens* is a Gram-negative soil bacterium that forms a symbiotic relationship with the *Heterorhabditis* nematode (Forst and Nealson 1996). This bacterium contains the *luxCDABE* operon, which encodes all of the proteins necessary for luminescence (Meighen 1993). *luxAB* encodes luciferase, the enzyme that catalyzes the oxidation of a long chain aldehyde and reduced flavin mononucleotide ($FMNH_2$) concomitantly reducing oxygen and resulting in the emission of blue-green light at a wavelength of 490nm (Meighen 1991). *luxCDE*, encodes the fatty acid reductase complex that consists of a reductase, transferase and synthetase respectively, and provides the long chain aldehyde substrate (Meighen 1991).

Previous studies have used a system whereby the reporter *luxAB* was incorporated into the bacterial genome and at a predetermined time, the exogenous substrate luciferin is added resulting in the emission of light (Hutter, Fischer et al. 2004). This type of reporter system has several limitations including its requirement to introduce the luciferin substrate to the cells. We were interested in developing a system which would eliminate the need to add in exogenous substrate in order to monitor promoter activity in real-time.

To this end, pSB2025 that contained the *luxABCDE* operon was used (Qazi, Counil et al. 2001). These *lux* genes were modified for use in Gram-positive bacteria by inserting ribosome binding sites optimized for expression in Gram-positive species upstream of *luxA, luxC* and *luxE* (Qazi, Counil et al. 2001). To allow for selection in *B. subtilis* 168, an erythromycin resistance cassette was inserted into pSB2025 using *XhoI* and *PstI* restriction sites, yielding pLuxErm (Figure 2.1).

The promoter region of each of the ten short-listed genes that were found to be transcriptionally activated upon TagD depletion were cloned into the multiple cloning site of pLuxErm. The candidate promoters were transcriptionally fused to the *lux* genes and introduced into *B. subtilis* using Campbell-type integration that conserves the native context of the surrounding genomic sequence. These reporter systems were introduced into *tagB, tagD* and *tagF* conditional mutant strains to monitor luminescence as each gene product was depleted. The reporter systems were also introduced into the wild-type *B. subtilis* strain to monitor their response to various antibiotics.

3.3 Teichoic Acid Depletion Studies

The activity of candidate reporter strains was initially studied in the *tagD* conditional mutant background. Reporter strains were grown in the presence and absence of xylose and growth and luminescence were monitored over a period of approximately 12 hours. The fold increase in luminescence was determined specifically for each reporter strain at different time points as the optimal response time varied between reporter strains (See Appendix I for a time-course response of each reporter strain to TagD-depletion). This variation in response time highlights the advantage of using a real-time reporting system. All of the reporter strains responded to TagD-depletion with at least a 2-fold increase in luminescence (Table 3.2). Reporter strains that demonstrated a 4-fold increase in luminescence or higher in the TagD-depletion studies were further characterized in *tagB* and *tagF* conditional mutant backgrounds. The majority of the reporter strains responded well to TagB- and TagF-depletion with over a 4 fold increase in luminescence (Table 3.2) (See appendix II and III for a time-course response of each reporter strain to TagB- and TagF-depletion). The results of the TagB-, TagD-, and TagF-depletion studies indicated that the P_{ywaC} reporter strain showed the most promise as a reporter of lesions in teichoic acid biosynthesis. Figure 3.2 depicts a typical response seen from the P_{ywaC} reporter strain to Tag protein depletion. An increase in luminescence was observed when TagD was depleted marked by a corresponding decrease in growth. Normalizing for changes in cell density resulted in a 12-fold increase in normalized luminescence (Table 3.2). To ensure that the luminescence observed with these reporter strains was due to transcriptional activation in response to a stress in their target pathway, a reporter system

using the promoter region of *serS*, a seryl-tRNA synthetase, was made. The expression of *serS* in the TagD microarray did not change between replete and deplete conditions proving to be an ideal candidate as a control promoter (See Appendix I, II and III for the time-course response of *serS* to TagB-, TagD-, and TagF-depletion).

In addition to testing the P_{ywaC} reporter system's response to Tag protein depletion, its response to the depletion of *yacN*, a gene involved in an isoprenoid biosynthetic pathway was measured. The 1-deoxy-d-xylulose 5-phosphate (DOXP) pathway and the mevalonate pathway are both used for isoprenoid biosynthesis with the DOXP pathway being the most commonly used pathway by eubacteria (Lichtenthaler 2000). IspF is an enzyme found in *E. coli* that is part of the DOXP pathway and catalyzes the cyclization of 4-diphosphocytidyl-2-*C*-methylerythritol-2-phosphate into 2-*C*-methyl-d-erythritol 2,4-cyclodiphosphate (MEC) (Herz, Wungsintaweekul et al. 2000). Previous studies have shown that the orthologue of this gene in *B. subtilis*, YacN is essential to the viability of this bacterium and the absence of this gene primarily impacts cell wall biosynthesis (Campbell and Brown 2002). The depletion of YacN in *B. subtilis* was tested in a strain containing the P_{ywaC} reporter system (EB1594). The *yacN* conditional mutant (EB323) was constructed essentially the same way as described above for the *tagB, tagD,* and *tagF* conditional mutants whereby the endogenous copy of the gene is replaced with a spectinomycin resistance cassette and a complementing copy of the gene is under the control of the xylose-inducible promoter at the *amyE* locus. The P_{ywaC} reporter system responded to the depletion of *yacN* with a 3-fold increase in normalized

luminescence after 480 minutes of growth (Table 3.2) (see Appendix IV for the response of the P_{ywaC} and P_{serS} reporter systems to *yacN* depletion).

Table 3.2 Response of reporter strains to TagB-, TagD-, TagF- and YacN-depletion.

Test Promoter	Fold increase in luminescence[a] between replete and deplete conditions			
	TagD	TagF	TagB	YacN
P_{ywaC}	12	20	4	3
P_{murB}	6	4.5	4	ND
P_{yhaU}	4	6	4.5	ND
P_{yktB}	4	6	2	ND
P_{yubB}	4	5	4	ND
P_{yvfT}	4.5	2.5	2	ND
P_{yqiW}	3	ND	ND	ND
P_{yfiB}	3	ND	ND	ND
P_{yxeI}	2	ND	ND	ND
P_{ydbO}	2	ND	ND	ND
P_{serS}	1	1	1	1

[a] Fold increase in Luminescence was calculated from the counts per second (CPS)/ OD_{600} at the optimal time point for each reporter strain.

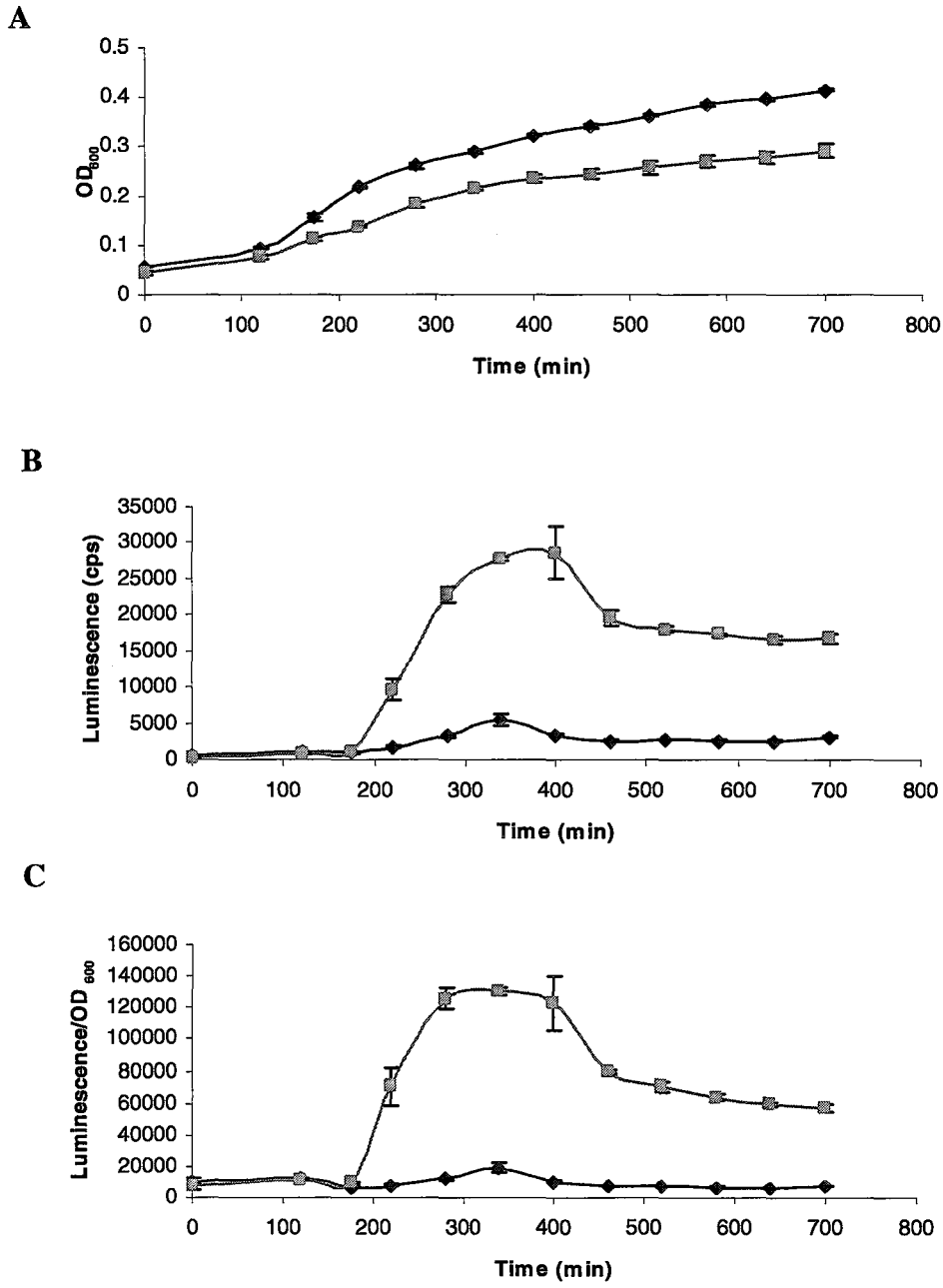


Figure 3.2. Effects of TagD depletion on the P_{ywaC} reporter strain in the *tagD* conditional mutant background (EB1402). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▨) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

3.4 Antibiotic Studies

To further characterize the top candidate reporter system, P_{ywaC} (EB1385), its response to various antibiotics in the wild-type *B. subtilis* 168 background was examined. The P_{ywaC} reporter strain (EB1385) was treated with fifteen antibiotics of various mechanisms of action. The minimum inhibitory concentration (MIC) of each antibiotic tested against the P_{ywaC} reporter strain (EB1385) was determined and concentrations two and four times above and below this concentration were used to assess the sensitivity of this reporter system. Antibiotics that target DNA, RNA and protein synthesis, did not transcriptionally activate the P_{ywaC} reporter strain (Figure 3.3). In contrast, the P_{ywaC} reporter strain (EB1385) responded well to the majority of cell wall antibiotics including bacitracin, fosfomycin, fosmidomycin, ramoplanin and vancomycin with at least an 8-fold increase in normalized luminescence at the concentration of antibiotic that caused the peak response from the reporter system (Figure 3.3). The P_{ywaC} reporter strain did not exhibit a strong response to the other cell wall antibiotics ampicillin, cefotaxime, cycloserine, and tunicamycin. However, while the concentrations 4-fold and 2-fold below the MIC of tunicamycin activate the reporter system approximately 4-fold, a concentration six times below the MIC was found to activate the reporter system 7-fold (Figure 3.4).

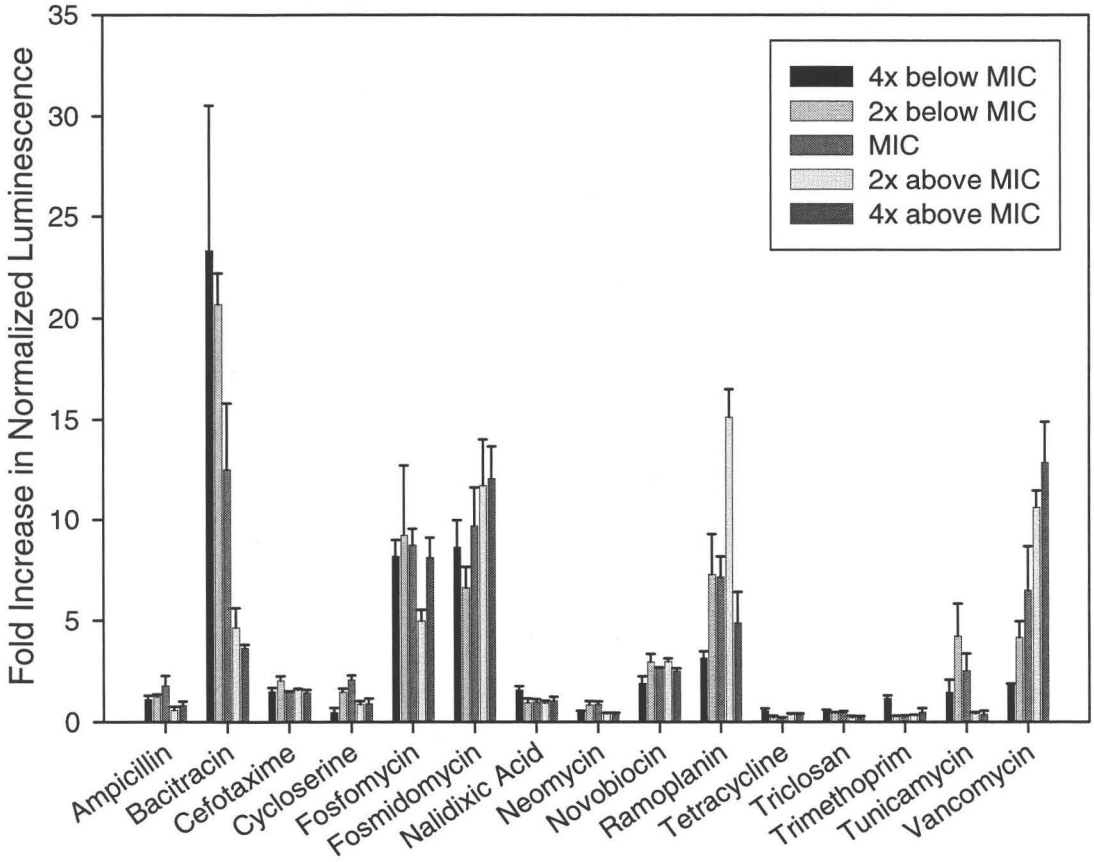


Figure 3.3. Effects of various antibiotics on the P_{ywaC} reporter strain in the wild-type *B. subtilis* 168 background. These strains were grown in LB supplemented with erythromycin. Fold increase in luminescence was calculated from the normalized luminescence (cps/OD_{600}) value of the response of the reporter strain to a particular compound after 3.5 hours of growth with the compound, divided by the normalized luminescence value of the low control (no antibiotic) at the same time point. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

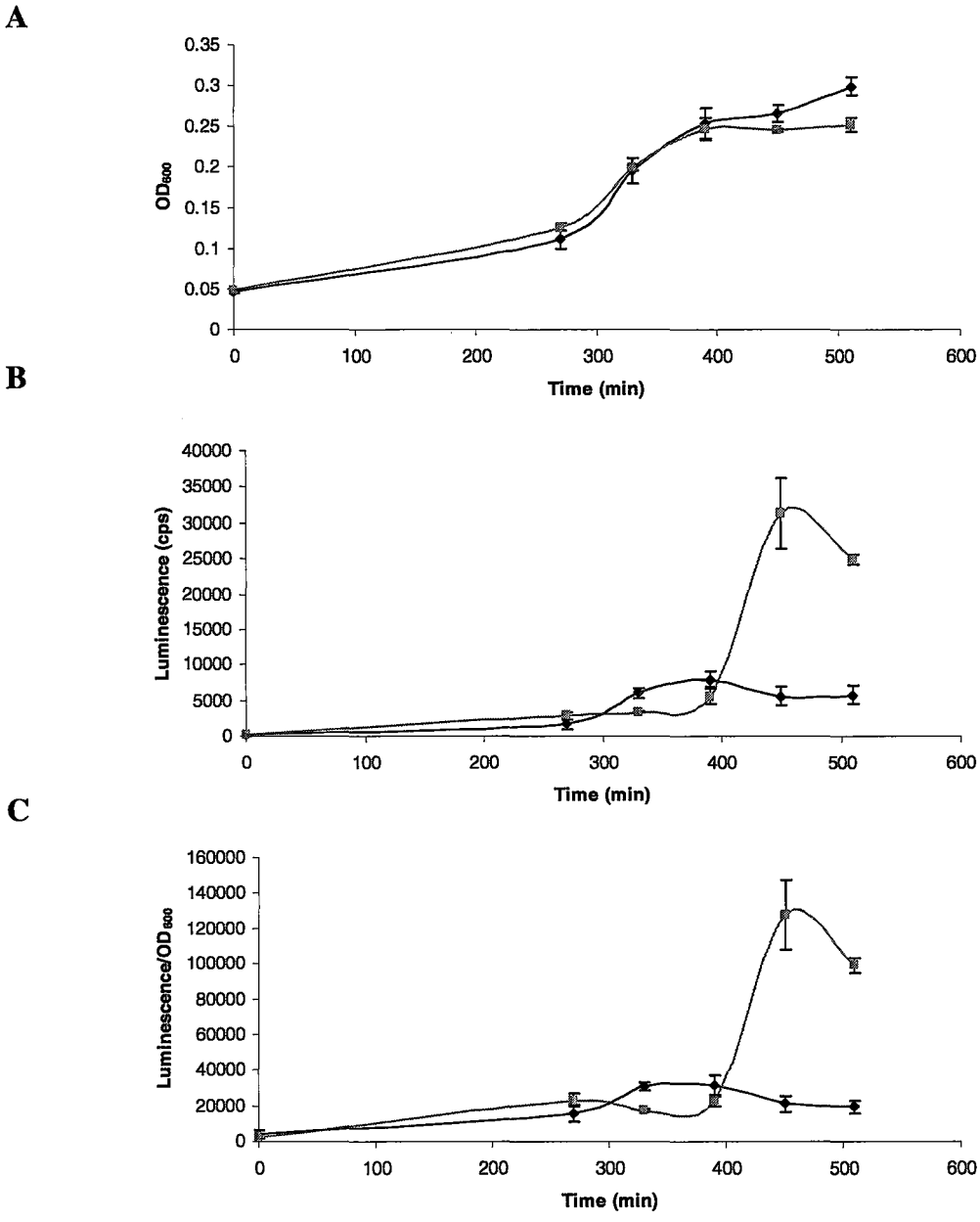


Figure 3.4 Effect of tunicamycin on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin in the presence (■) and absence (◆) of 1 μg/ml tunicamycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Tunicamycin was added to the cells after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

3.5 P_{ywaC} is a reporter that responds to cell wall active compounds

The luminescence studies described above provided evidence that the P_{ywaC} reporter strain (EB1385) is a sensitive reporter system that is able to respond to lesions in both peptidoglycan and teichoic acid biosynthesis. We next evaluated the P_{ywaC} reporter strain for its amenability for high throughput screening. A Z' value was calculated for the P_{ywaC} reporter strain using bacitracin treatment as the high control and DMSO as the low control. The Z' value is a statistical measure that determines the robustness of an assay (Zhang, Chung et al. 1999). From the previous luminescence studies, bacitracin was found to elicit the best response from the P_{ywaC} reporter strain (Figure 3.3) and that DMSO did not affect the P_{ywaC} reporter strain (Figure 3.5). Two hundred and forty replicates of both the positive and negative control were used to determine a Z' value of 0.6 for the P_{ywaC} reporter strain (Figure 3.6).

The P_{ywaC} reporter strain was used in a continuous, real-time reporter assay to test its response to compounds of known pharmacology (Prestwick chemicals) as a proof-of-principle screen (Figure 3.7). Of the 1120 compounds tested, 31 were identified that impinged upon peptidoglycan or teichoic acid biosynthesis (Table 3.3), as judged by P_{ywaC} induction. A range of induction was observed with vancomycin, cloxacillin, and flucloxacillin, compounds that transcriptionally activated the P_{ywaC} reporter strain 10-fold or higher.

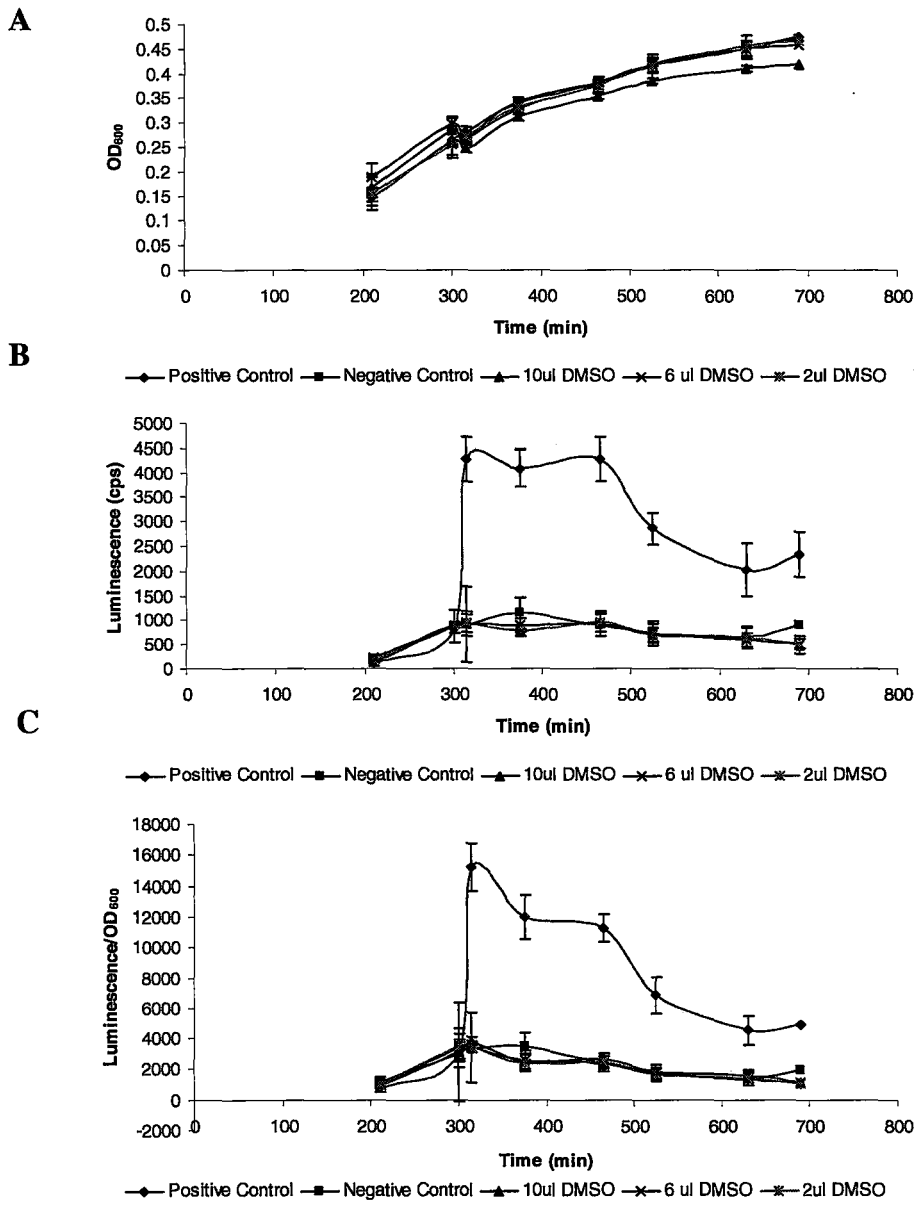


Figure 3.5. Effect of dimethyl sulfoxide (DMSO) on the P_{ywaC} reporter strain in the wildtype background (EB1385). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Bacitracin (64 μg/ml) and No drug were used as the positive and negative controls respectively. Compounds were added to the cells after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

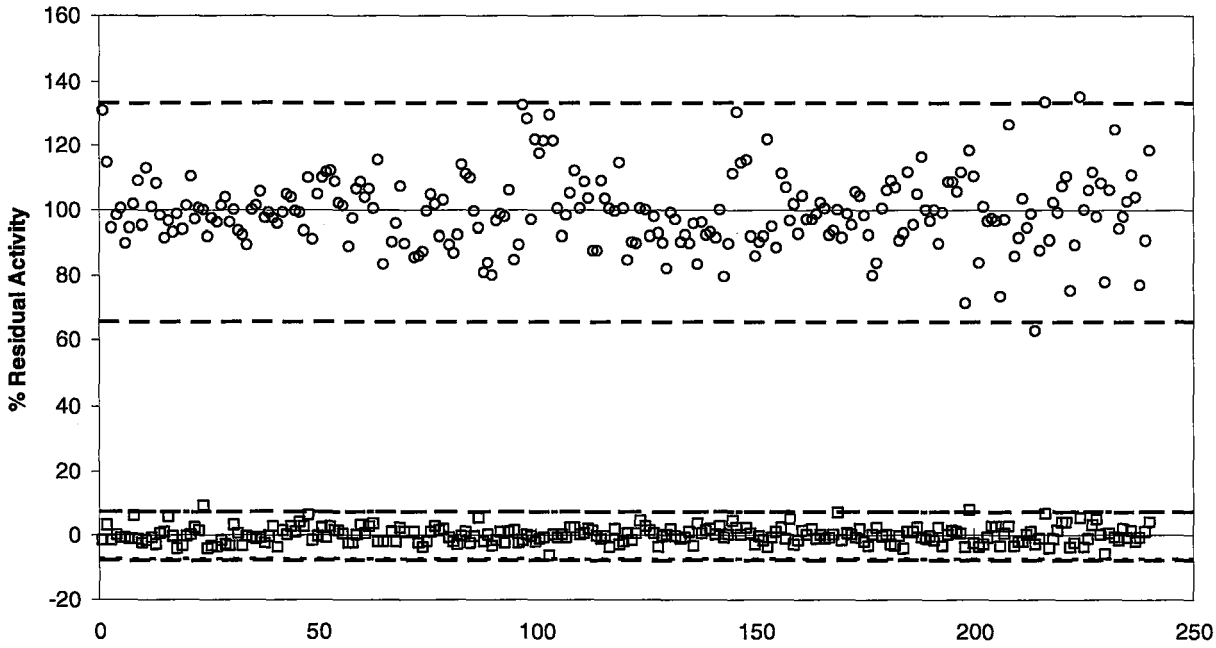


Figure 3.6 Evaluating the amenability of the P_{ywaC} reporter system for use in high-throughput screening (EB1385). The residual activity of the high (EB1385 treated with bacitracin) and the low (EB1385 treated with DMSO) controls were plotted for each replicate. The dashed line (– –) represents three standard deviations from the mean of both the high (O) and the low (□) controls. The screening window is located between the inner dashed lines for the high and low controls at approximately 65 and 8 % residual activity respectively. The quality of the screen was assessed using the statistical parameter Z' and was found to be 0.6.

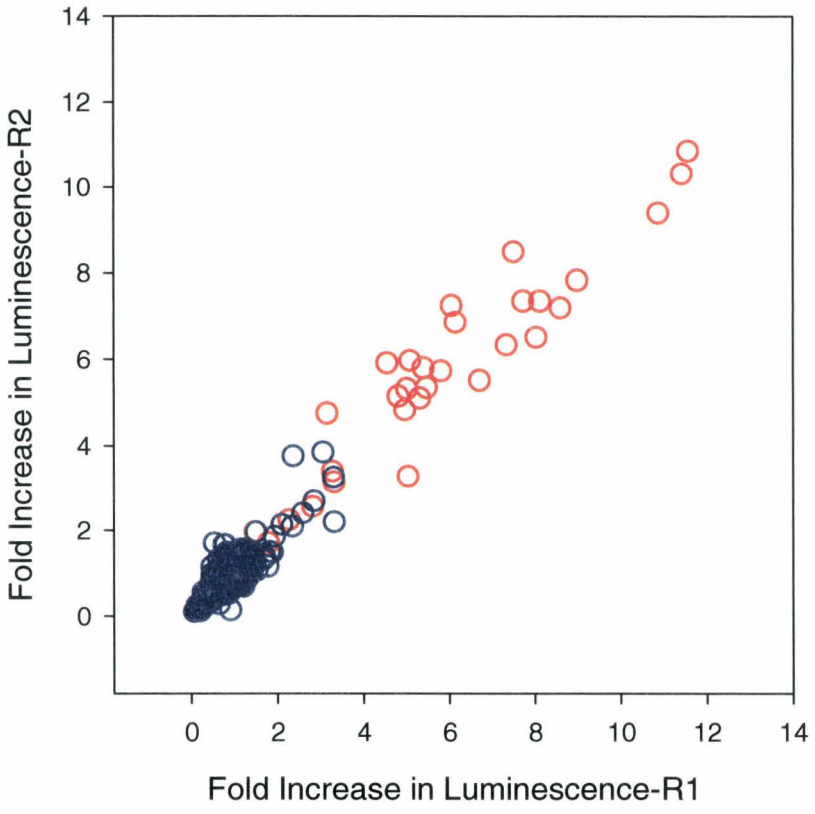


Figure 3.7. Identification of compounds that interact with P_{ywaC} reporter system from a small molecule library. Replicate plot showing the fold increase in luminescence of the P_{ywaC} reporter strain (EB1385) in response to compounds in the Prestwick library (10μM). Fold increase in luminescence was calculated from the normalized luminescence (cps/OD_{600}) value of the maximal response of the reporter strain to a particular compound, divided by the normalized luminescence value of the low control (DMSO). Points in the upper right corner indicate compounds that transcriptionally activated the P_{ywaC} reporter system. Red points represent cell wall compounds while blue points represent all other compounds in library.

Table 3.3. Compounds from the small molecule screen found to transcriptionally activate the P_{ywaC} reporter system.

Compound	Average Fold Increase In Normalized Luminescence[a]	Pharmacology
Vancomycin	13	Antibacterial
Flucloxacillin	11	Antibacterial
Cloxacillin	10	Antibacterial
Cephalonium	8.5	Antibacterial
Meropenem	8	Antibacterial
Moxalactam sodium salt	8	Antibacterial
Naficillin sodium salt	8	Antibacterial
Cefoperazone dihydrate	7.5	Antibacterial
Cefamandole sodium salt	7	Antibacterial
Cefoxitin sodium salt	7	Antibacterial
Imipenem	7	Antibacterial
Loracarbef	6.5	Antibacterial
Cefaclor	6	Antibacterial
Cefotiam hydrochloride	6	Antibacterial
Cefepime hydrochloride	5.5	Antibacterial
Cefuroxime sodium salt	5.5	Antibacterial
Dicloxacillin	5.5	Antibacterial
Cefazolin sodium salt	5	Antibacterial
Cefmetazole sodium salt	5	Antibacterial
Ceforanide	5	Antibacterial
Cefotaxime sodium salt	5	Antibacterial
Cefotetan	5	Antibacterial
Bacitracin	4	Antibacterial
Cephalothin sodium salt	4	Antibacterial
Homosalate	3.5	Tool for uv screen
Cefixime	3	Antibacterial
Ceftazidime pentahydrate	3	Antibacterial
Chlorohexidine	3	Antiseptic
Hexetidine	3	Antifungal
Methyl Benzethoniam Chloride	3	Antibacterial
Betulin	2.5	Antineoplastic Antiinflammatory

[a] Fold increase in Luminescence was calculated from the counts per second (CPS)/ OD_{600} at the optimal time point for each reporter strain.

The Prestwick library contains 171 antibiotics, 45 of which are known to target the cell wall. In total, 26 of the 45 antibiotics targeting the cell wall caused a marked decrease in cell growth of the P_{ywaC} reporter strain while the remainder of the cell wall antibiotics did not affect cell growth. Of the 31 hits, 26 compounds have known mechanisms of action targeting cell wall biosynthesis, with 22 of these cell wall active compounds impacting the growth of the P_{ywaC} reporter strain (Table 3.4). The other four cell wall active compounds that did not impede cell growth further corroborate the sensitivity of this reporter strain (Table 3.5). The cell wall antibiotics that did not affect cell growth and did not transcriptionally activate the P_{ywaC} reporter strain may be due to their inability to penetrate the *B. subtilis* cell. Interestingly, the five compounds found to transcriptionally activate the reporter strain and are not cell wall active compounds have an unknown mechanism of action (Table 3.6). Two of these compounds, chlorohexidine and methyl benzethonium chloride, impact the growth of the P_{ywaC} reporter strain (EB1385) by causing a decrease in cell density while simultaneously increasing luminescence. The three remaining hits, betulin, hexetidine and homosalate, do not affect cell growth however they do cause an approximate 3-fold increase in normalized luminescence, which is also seen with chlorohexidine and methyl benzethonium chloride.

Table 3.4. Cell wall antibiotics in the Prestwick database found to affect cell growth. Antibiotics in bold indicate antibiotics that transcriptionally activated the P_{ywaC} reporter system in the proof of principle screen.

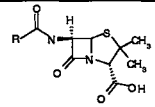
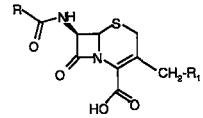
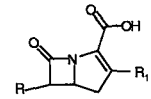
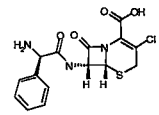
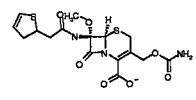
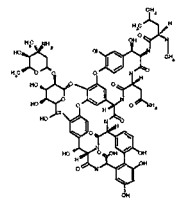
Name	Structure	Mechanism of Action	Hit
Penicillin			
Cloxacillin		**Transpeptidase inhibitor**	**Yes**
Dicloxacillin		**Transpeptidase inhibitor**	**Yes**
Flucloxacillin		**Transpeptidase inhibitor**	**Yes**
Naficillin sodium salt		**Transpeptidase inhibitor**	**Yes**
Cephalosporin			
First generation			
Cefotetan		**Transpeptidase inhibitor**	**Yes**
Cefazolin sodium salt		**Transpeptidase inhibitor**	**Yes**
Cephadroxil		Transpeptidase inhibitor	No
Cephalexin monohydrate		Transpeptidase inhibitor	No
Cephalonium		**Transpeptidase inhibitor**	**Yes**
Cephalothin sodium salt		**Transpeptidase inhibitor**	**Yes**
Second generation			
Cefaclor		**Transpeptidase inhibitor**	**Yes**
Cefamandole sodium salt		**Transpeptidase inhibitor**	**Yes**
Cefmetazole sodium salt		**Transpeptidase inhibitor**	**Yes**
Ceforanide		**Transpeptidase inhibitor**	**Yes**
Cefuroxime sodium salt		**Transpeptidase inhibitor**	**Yes**
Third generation			
Cefoperazone sodium salt		**Transpeptidase inhibitor**	**Yes**
Cefotaxime sodium salt		**Transpeptidase inhibitor**	**Yes**
Cefotiam hydrochloride		**Transpeptidase inhibitor**	**Yes**
Moxolactam sodium salt		**Transpeptidase inhibitor**	**Yes**
Carbapenem			
Meropenem		**Transpeptidase inhibitor**	**Yes**
Imipenem		**Transpeptidase inhibitor**	**Yes**
Carbacephem			
Loracarbef		**Transpeptidase inhibitor**	**Yes**
Cephamycin			
Cefoxitin sodium salt		**Transpeptidase inhibitor**	**Yes**
Glycopeptide			
Vancomycin		**Transpeptidase inhibitor**	**Yes**

Table 3.5. Cell wall antibiotics in the Prestwick database found not to affect cell growth. Antibiotics in bold indicate antibiotics that transcriptionally activated the P_{ywaC} reporter system in the proof of principle screen.

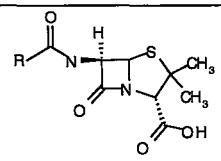
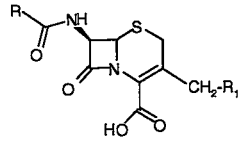
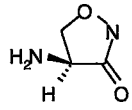
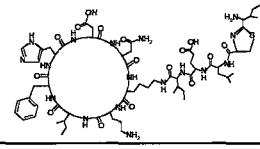
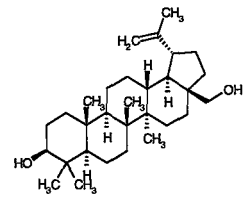
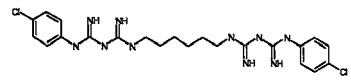
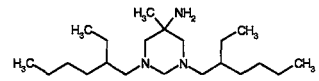
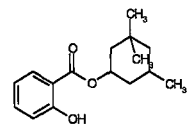
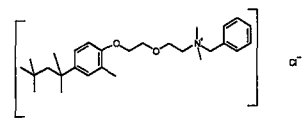
Name	Structure	Mechanism of Action	Hit
Penicillin			
Ampicillin trihydrate		Bacterial transpeptidase inhibitor	No
Amoxicillin		Bacterial transpeptidase inhibitor	No
Azlocillin sodium salt		Bacterial transpeptidase inhibitor	No
Aztreonam		Bacterial transpeptidase inhibitor	No
Bacampicillin hydrochloride		Bacterial transpeptidase inhibitor	No
Ceftazidime pentahydrate		**Bacterial transpeptidase inhibitor**	**Yes**
Cyclacillin		Bacterial transpeptidase inhibitor	No
Metampicillin sodium salt		Bacterial transpeptidase inhibitor	No
Phenethicillin potassium salt		Bacterial transpeptidase inhibitor	No
Piperacillin sodium salt		Bacterial transpeptidase inhibitor	No
Pivampicillin		Bacterial transpeptidase inhibitor	No
Pivmecillinam hydrochloride		Bacterial transpeptidase inhibitor	No
Talampicillin hydrochloride		Bacterial transpeptidase inhibitor	No
Ticarcillin sodium salt		Bacterial transpeptidase inhibitor	No
Cephalosporin			
First generation			
Cefepime hydrochloride		**Bacterial transpeptidase inhibitor**	**Yes**
Cephalosporanic acid, 7-amino		Bacterial transpeptidase inhibitor	No
Third generation			
Cefixime		**Bacterial transpeptidase inhibitor**	**Yes**
Cefsoludin sodium salt		Bacterial transpeptidase inhibitor	No
D-ala racemase/ D-ala ligase			
Cycloserine		D-ala racemase/D-ala ligase inhibitor	No
Polypeptide			
Bacitracin		**Isoprenoid biosynthesis inhibitor**	**Yes**

Table 3.6 Compounds from the small molecule library found to transcriptionally activate the P_{ywaC} reporter system (EB1385). These compounds have an unknown mechanism of action.

Name	Pharmacology	Structure
Betulin	Antineoplastic Antiinflammatory	
Chlorohexidine	Antiseptic	
Hexetidine	Antifungal	
Homosalate	Tool for uv screen	
Methyl Benzethoniam Chloride	Antibacterial	

CHAPTER 4 – DISCUSSION

The limited number of physiological pathways in bacteria that are currently targeted by antibiotics has triggered a search for novel processes in the bacterial cell which may be exploited as therapeutic targets. It should be appreciated, that the physiological processes that serve as current targets are complex and perhaps yet contain good targets. For example, cell wall active antibiotics almost exclusively inhibit peptidoglycan synthesis. However, the cell wall of Gram-positives is known to be comprised of anionic polymers that are covalently attached to peptidoglycan. Teichoic acid is one such structure which has been investigated for its essentiality in Gram-positive bacteria. Many of the teichoic acid biosynthetic genes have been shown to be essential to *B. subtilis* (Bhavsar, Beveridge et al. 2001; Bhavsar, Erdman et al. 2004) and thus teichoic acid biosynthesis is an interesting pathway to study for its potential as an antimicrobial target. Since the peptidoglycan biosynthetic pathway is the pathway most commonly targeted by current antimicrobials, it would be ideal to design a reporter system unique to the teichoic acid biosynthetic pathway to probe for molecules that impinged upon this pathway. Designing a reporter system that responds specifically to lesions in only one pathway in the bacterial cell wall however, may prove to be a difficult task as cell wall processes may be intimately linked.

The microarray analysis of TagD-depleted cells using the *tagD* conditionally complemented mutant (EB240), resulted in the identification of 180 genes that were transcriptionally up-regulated in response to this depletion. The promoter region of ten of

these genes were used to study cell wall lesions whereby half of these reporter systems were predicted to respond to teichoic acid depletion and the remainder were predicted to respond to lesions in both teichoic acid and peptidoglycan synthesis. Characterization of these reporter systems in *tag* mutant backgrounds resulted in the identification of the candidate promoter to be used for further studies, the promoter region of *ywaC*. While the other reporter systems did respond to Tag protein depletion, the P_{ywaC} reporter system had the overall best response to Tag protein depletion and thus was chosen for further characterization. The response of the P_{ywaC} reporter system was also characterized in the *yacN* mutant background, a gene involved in the DOXP pathway which is linked to cell wall biosynthesis (Campbell and Brown 2002), and was found to respond well to this gene's depletion.

The experiments described herein provide evidence that the P_{ywaC} reporter system responds to lesions in cell wall biosynthesis. Upon a closer examination of the data, it could be hypothesized that the P_{ywaC} reporter system may be responding to the levels of undecaprenol-phosphate in the cell, a shared building block between peptidoglycan and teichoic acid biosynthesis. Experiments from the 1970's suggest that there is an overlap between peptidoglycan and teichoic acid synthesis through the lipid-linked carrier, undecaprenol-phosphate. In 1972, Anderson *et. al.*, conducted *in vitro* studies and showed that peptidoglycan synthesis was inhibited with the addition of teichoic acid precursors and teichoic acid synthesis was inhibited with the addition of peptidoglycan precursors in *B. subtilis* and *Bacillus licheniformis* (Anderson, Hussey et al. 1972). This

study supports the hypothesis that undecaprenol-phosphate is a shared building block between peptidoglycan and teichoic acid biosynthesis and when this lipid-linked carrier is prevented from re-entering a common pool, it can lead to the inhibition of both teichoic acid and peptidoglycan synthesis. Through the characterization of the P_{ywaC} reporter system, it appears that this reporter responds to a stress to teichoic acid biosynthesis as well as responding to a subset of molecules impinging on peptidoglycan synthesis and thus it is plausible that the P_{ywaC} reporter system is an exquisite reporter of undecaprenol-phosphate recycling, providing an integral measure for responding to cell wall stress.

Using the candidate reporter system $P_{ywaC,}$, its response to antibiotics of various mechanism of action was explored to determine its specificity for lesions in the cell wall. Cell wall active antibiotics were limited to those that affect peptidoglycan synthesis as to date, there are no widely accepted drugs to study teichoic acid biosynthesis. As anticipated, antibiotics that did not impinge on cell wall biosynthesis did not activate the reporter system. However, antibiotics targeting various areas of the cell wall including bacitracin, fosfomycin, fosmidomycin, ramoplanin and vancomycin, activated the reporter system but interestingly, the P_{ywaC} reporter system did not respond well to the cell wall antibiotics ampicillin, cefotaxime and cycloserine.

Of the antibiotics tested, bacitracin elicited the best response from the P_{ywaC} reporter system. Bacitracin inhibits the de-phosphorylation of undecaprenol-pyrophosphate to yield undecaprenol-phosphate, the membrane-embedded carrier for teichoic acid and peptidoglycan biosynthesis. Fosmidomycin is a cell wall active antibiotic that activated the reporter system with an 8-fold increase in normalized luminescence and influences isoprenoid biosynthesis through the inhibition of IspC. Vancomycin binds to D-alanine-D-alanine of the pentapeptide unit of peptidoglycan, preventing transpeptidation from occurring in peptidoglycan synthesis. Through binding to D-alanine, vancomycin causes a steric blockade which indirectly affects transglycosylation by interfering with the penicillin binding proteins (Walsh 2003). Ramoplanin also affects transglycosylation by blocking the polymerization of Lipid II (Lo 2000). The peptidoglycan unit that is added to the growing strand of peptidoglycan is a disaccharyl-pentapeptide, which is attached to C_{55}-isoprenyl phosphate (lipid II). Upon transglycosylation, the phosphodiester bond between the disaccharyl-pentapeptide and undecaprenol-phosphate gets cleaved, allowing the disaccharyl-pentapeptide to be added to the growing peptidoglycan layer (Walsh 2003). The inhibition of transglycosylation by various antibiotics affects C_{55}-isoprenyl phosphate by preventing its liberation from the disaccharyl-pentapeptide and thus it cannot be recycled for subsequent peptidoglycan synthesis.

The effects of the aforementioned antibiotics either directly, or indirectly, affect the levels of undecaprenol-phosphate by preventing its regeneration. Tunicamycin, a cell

wall active antibiotic thought to inhibit translocase I, encoded by *mraY,* catalyzes the addition of the MurNAc-pentapeptide to undecaprenol-phosphate yielding lipid intermediate I (Kimura and Bugg 2003). This antibiotic may also affect undecaprenol-phosphate levels in the cell but it was found to cause only a modest increase in transcription of the P_{ywaC} reporter system with a 4-fold increase in normalized luminescence. Concentrations above the MIC do not activate the reporter system however in addition to testing concentrations 2 and 4 times above and below the MIC, a concentration 6-fold below the MIC was tested and found to activate the reporter system 7-fold. This shows that only a low concentration of tunicamycin is needed to activate the reporter system and that concentrations above this concentration, do not have a strong effect on the reporter system. Fosfomycin targets MurA, the first committed step in the peptidoglycan biosynthetic pathway and was shown to activate the reporter system. However, the P_{ywaC} reporter system does not appear to respond well to cycloserine, a cell wall antibiotic which targets D-alanine racemase and D-ala-D-ala ligase (Pinho and Errington 2005) (Figure 3.3). The inhibition of D-alanine racemase and D-ala-D-ala ligase may not affect peptidoglycan transglycosylation which would allow undecaprenol-phosphate to be regenerated for use in teichoic acid and peptidoglycan synthesis. Ampicillin and cefotaxime, both β-lactam antibiotics, inhibit the transpeptidation step of peptidoglycan synthesis which prevents the pentapeptide bridge from forming. The reporter system did not respond well to these antibiotics however this type of inhibition, may not affect the regeneration of undecaprenol-phosphate levels in the cell.

It is interesting to note that compounds directly affecting undecaprenol-phosphate levels are only needed in a low concentration to impact the activity of the reporter system whereas compounds indirectly affecting these levels are needed at a higher concentration to elicit a response from the reporter system (Figure 3.3). In particular, bacitracin does not require concentrations above the MIC to activate the reporter system. Bacitracin directly affects undecaprenol-phosphate levels by inhibiting the de-phosphorylation of C_{55}-isoprenyl pyrophosphate, preventing the regeneration of this membrane-embedded carrier required for both peptidoglycan and teichoic acid biosynthesis (Stone and Strominger 1971). In contrast, vancomycin does not act directly at the site of undecaprenol-phosphate recycling but instead works by preventing peptidoglycan transpeptidation by binding to D-alanine on the pentapeptide unit. Accordingly, concentrations above the MIC of vancomycin may be required to activate the P_{ywaC} reporter system as inhibition of the undecaprenol-phosphate recycling pathway by vancomycin would be an indirect result of the inhibition of transpeptidation. The various concentrations of fosfomycin and fosmidomycin have approximately the same impact on reporter activity with the output of luminescence remaining at a relatively consistent level over the various antibiotic concentrations. Not only do these differences in response from the P_{ywaC} reporter system herald the sensitivity of this reporter system as molecules need not impinge on cell growth in order to elicit a response from the reporter system, but it also supports the idea that the promoter region of *ywaC* may respond to undecaprenol phosphate levels in the cell.

The proof of principle screen conducted has provided further evidence that the P_{ywaC} reporter system is a reporter of cell wall stress. A Z' value of 0.6 was calculated for the P_{ywaC} reporter system indicating that it was amenable for high throughput screening. The response of the P_{ywaC} reporter system was tested against 1120 FDA approved drugs from the Prestwick library. The results of this screen identified 31 compounds that activated the P_{ywaC} reporter system. Twenty-six of these compounds are known to target the cell wall, supporting the hypothesis that the P_{ywaC} reporter system responds to lesions in the cell wall. The promoter-reporter system used to conduct the small molecule screen is advantageous for high throughput screening in that it allows you to monitor *in vivo* activity over time through the *luxABCDE* operon. It also gives a clue as to the mechanism of action of lead compounds as compounds perturbing cell wall processes can potentially activate the P_{ywaC} reporter system.

The gene *ywaC* is an uncharacterized protein that resembles a GTP pyrophosphokinase. These enzymes synthesize ppGpp and permit cell signaling during the stringent response to amino acid starvation. Previous studies have revealed that *ywaC* is a member of the σ^W regulon (Cao, Kobel et al. 2002). σ^W is thought to regulate genes that protect the cell from compounds that impair cell wall synthesis (Cao, Kobel et al. 2002). There are approximately 60 genes in *B. subtilis* 168 that are involved in the σ^W regulon and they are specifically regulated transcriptionally in response to cell wall active compounds and not to compounds that target protein or DNA synthesis (Cao, Kobel et al. 2002). While it may have been anticipated that the P_{ywaC} reporter system would respond

to lesions in peptidoglycan synthesis, it was not anticipated to respond to lesions in teichoic acid synthesis. Nevertheless, the results indicate that it does respond to this stimuli and leaves open the question of further regulation of teichoic acid biosynthesis by σ^W. These findings make the P_{ywaC} reporter strain a two pathway specific reporter that can respond not only to lesions in peptidoglycan synthesis but also teichoic acid synthesis which could be attributed to its potential specificity for undecaprenol-phosphate recycling.

4.1 Concluding Remarks

In summary, the P_{ywaC} reporter system is a reporter system that responds to lesions in both peptidoglycan and teichoic acid biosynthesis. While there are many antibiotics available to date that target peptidoglycan, teichoic acid has yet to be used as a therapeutic target. The P_{ywaC} reporter strain is a genetically engineered *B. subtilis* strain that can be used in a cell-based screening assay. Cell-based screening assays have been deemed problematic in the past due to their inability to identify the mechanism of action of a given active compound however they can discriminate against compounds that are not antimicrobials. By integrating the P_{ywaC} reporter system into the *B. subtilis* 168 wild-type genome, a screen using this reporter system selects not only for antimicrobial compounds, but also gives an indication of the mechanism of action of the compound. The small molecule screen described herein validates the use of the P_{ywaC} reporter strain for high throughput screening. The library of compounds screened represents a proof of principle for larger-scale efforts using the P_{ywaC} reporter strain to find new compounds that are growth inhibitory in their mode of action on cell wall synthesis. The identification of such molecules would lay the groundwork for developing novel antimicrobials specific to teichoic acid biosynthesis and may represent an important step in combating antibiotic resistance.

References

Anderson, R. G., H. Hussey, et al. (1972). "The mechanism of wall synthesis in bacteria. The organization of enzymes and isoprenoid phosphates in the membrane." Biochem J **127**(1): 11-25.

Archibald, A., Hancock, IC (1993). Cell wall structure, synthesis and turnover. *Bacillus subtilis* and other gram-positive bacteria. A. Sonenshein, Hoch, JA, Losick, R. Washington, DC, American Society for Microbiology: 381-410.

Bhavsar, A. P., T. J. Beveridge, et al. (2001). "Precise deletion of *tagD* and controlled depletion of its product, glycerol 3-phosphate cytidylyltransferase, leads to irregular morphology and lysis of *Bacillus subtilis* grown at physiological temperature." J Bacteriol **183**(22): 6688-6693.

Bhavsar, A. P. and E. D. Brown (2006). "Cell wall assembly in *Bacillus subtilis*: how spirals and spaces challenge paradigms." Mol Microbiol **60**(5): 1077-1090.

Bhavsar, A. P., L. K. Erdman, et al. (2004). "Teichoic acid is an essential polymer in *Bacillus subtilis* that is functionally distinct from teichuronic acid." J Bacteriol **186**(23): 7865-7873.

Bhavsar, A. P., R. Truant, et al. (2005). "The TagB protein in *Bacillus subtilis* 168 is an intracellular peripheral membrane protein that can incorporate glycerol phosphate onto a membrane-bound acceptor *in vitro*." J Biol Chem **280**(44): 36691-36700.

Brandt, C. and D. Karamata (1987). "Thermosensitive *Bacillus subtilis* mutants which lyse at the non-permissive temperature." J Gen Microbiol **133**(5): 1159-1170.

Briehl, M., Pooley, H, Karamata, D (1989). "Mutants of *Bacillus subtilis* 168 thermosensitive for growth and wall teichoic acid synthesis." J. Gen. Microbiol. **135**: 1325-1334.

Campbell, T. L. and E. D. Brown (2002). "Characterization of the depletion of 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase in *Escherichia coli* and *Bacillus subtilis*." J Bacteriol **184**(20): 5609-5618.

Cao, M., P. A. Kobel, et al. (2002). "Defining the *Bacillus subtilis* sigma(W) regulon: a comparative analysis of promoter consensus search, run-off transcription/macroarray analysis (ROMA), and transcriptional profiling approaches." J Mol Biol **316**(3): 443-457.

Cutting, S., Youngman, P (1994). in Methods for General and Molecular Bacteriology. R. G. E. Murray, Krieg, N. R., Wood, W. A., and Gerhardt, P., eds, American Society for Microbiology: 348-364.

D'Elia, M. A., M. P. Pereira, et al. (2006). "Lesions in teichoic acid biosynthesis in *Staphylococcus aureus* lead to a lethal gain of function in the otherwise dispensable pathway." J Bacteriol **188**(12): 4183-4189.

Fischer, H. P., N. A. Brunner, et al. (2004). "Identification of antibiotic stress-inducible promoters: a systematic approach to novel pathway-specific reporter assays for antibacterial drug discovery." Genome Res **14**(1): 90-98.

Forst, S. and K. Nealson (1996). "Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp." Microbiol Rev **60**(1): 21-43.

Francis, K. P., D. Joh, et al. (2000). "Monitoring bioluminescent *Staphylococcus aureus* infections in living mice using a novel *luxABCDE* construct." Infect Immun **68**(6): 3594-3600.

Herz, S., J. Wungsintaweekul, et al. (2000). "Biosynthesis of terpenoids: YgbB protein converts 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate to 2C-methyl-D-erythritol 2,4-cyclodiphosphate." Proc Natl Acad Sci U S A **97**(6): 2486-2490.

Hutter, B., C. Fischer, et al. (2004). "Panel of *Bacillus subtilis* reporter strains indicative of various modes of action." Antimicrob Agents Chemother **48**(7): 2588-2594.

Hutter, B., C. Schaab, et al. (2004). "Prediction of mechanisms of action of antibacterial compounds by gene expression profiling." Antimicrob Agents Chemother **48**(8): 2838-2844.

Kimura, K. and T. D. Bugg (2003). "Recent advances in antimicrobial nucleoside antibiotics targeting cell wall biosynthesis." Nat Prod Rep **20**(2): 252-273.

Lichtenthaler, H. K. (2000). "Non-mevalonate isoprenoid biosynthesis: enzymes, genes and inhibitors." Biochem Soc Trans **28**(6): 785-789.

Livermore, D. M. (2004). "The need for new antibiotics." Clin Microbiol Infect **10 Suppl 4**: 1-9.

Lo, M., Men, H Branstrom, A, Helm, J, Yao, N, Goldman, R, Walker, S (2000). "A new mechanism of action proposed for ramoplanin." J. Am. Chem. Soc. **122**: 3540-3541.

Meighen, E. A. (1991). "Molecular biology of bacterial bioluminescence." Microbiol Rev **55**(1): 123-142.

Meighen, E. A. (1993). "Bacterial bioluminescence: organization, regulation, and application of the *lux* genes." Faseb J **7**(11): 1016-1022.

Monaghan, R. L. and J. F. Barrett (2006). "Antibacterial drug discovery--then, now and the genomics future." Biochem Pharmacol **71**(7): 901-909.

Park, Y. S., T. D. Sweitzer, et al. (1993). "Expression, purification, and characterization of CTP:glycerol-3-phosphate cytidylyltransferase from *Bacillus subtilis*." J Biol Chem **268**(22): 16648-16654.

Pinho, M. G. and J. Errington (2005). "Recruitment of penicillin-binding protein PBP2 to the division site of Staphylococcus aureus is dependent on its transpeptidation substrates." Mol Microbiol **55**(3): 799-807.

Pooley, H. M., F. X. Abellan, et al. (1991). "A conditional-lethal mutant of *Bacillus subtilis* 168 with a thermosensitive glycerol-3-phosphate cytidylyltransferase, an enzyme specific for the synthesis of the major wall teichoic acid." J Gen Microbiol **137**(4): 921-928.

Powers, J. H. (2004). "Antimicrobial drug development--the past, the present, and the future." Clin Microbiol Infect **10 Suppl 4**: 23-31.

Pucci, M. J. (2006). "Use of genomics to select antibacterial targets." Biochem Pharmacol **71**(7): 1066-1072.

Qazi, S. N., E. Counil, et al. (2001). "*agr* expression precedes escape of internalized *Staphylococcus aureus* from the host endosome." Infect Immun **69**(11): 7074-7082.

Rosamond, J. and A. Allsop (2000). "Harnessing the power of the genome in the search for new antibiotics." Science **287**(5460): 1973-1976.

Sambrook, J., Fritsch, EF., Maniatis, T (1989). Molecular Cloning: A laboratory Manual, 2nd Ed. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.

Schertzer, J. W. and E. D. Brown (2003). "Purified, recombinant TagF protein from *Bacillus subtilis* 168 catalyzes the polymerization of glycerol phosphate onto a membrane acceptor in vitro." J Biol Chem **278**(20): 18002-18007.

Spring, D. R. (2005). "Chemical genetics to chemical genomics: small molecules offer big insights." Chem Soc Rev **34**(6): 472-482.

Stone, K. J. and J. L. Strominger (1971). "Mechanism of action of bacitracin: complexation with metal ion and C 55 -isoprenyl pyrophosphate." Proc Natl Acad Sci U S A **68**(12): 3223-3227.

Tenover, F. C. (2006). "Mechanisms of antimicrobial resistance in bacteria." Am J Infect Control **34**(5 Suppl): S3-S10.

Vagner, V., E. Dervyn, et al. (1998). "A vector for systematic gene inactivation in Bacillus subtilis." Microbiology **144 (Pt 11)**: 3097-3104.

Walsh, C. (2000). "Molecular mechanisms that confer antibacterial drug resistance." Nature **406**(6797): 775-781.

Walsh, C. (2003). Glycopeptide antibiotics act by complexing the un-cross-linked peptide strands and blocking transpeptidation. Antibiotics: Actions, Origins, Resistance. Washington, D.C., American Society For Microbiology: 45-48.

Walsh, C. (2003). Three phases of peptidoglycan enzymatic assembly: cytoplasmic, membrane-associated, and extracytoplasmic. Antibiotics: Actions, Origins, Resistance. Washington, D.C., American Society For Microbiology: 26-32.

Weidenmaier, C., J. F. Kokai-Kun, et al. (2004). "Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections." Nat Med **10**(3): 243-245.

Zhang, J. H., T. D. Chung, et al. (1999). "A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays." J Biomol Screen **4**(2): 67-73.

Appendix 5.1: Response of reporter strains to TagD-depletion

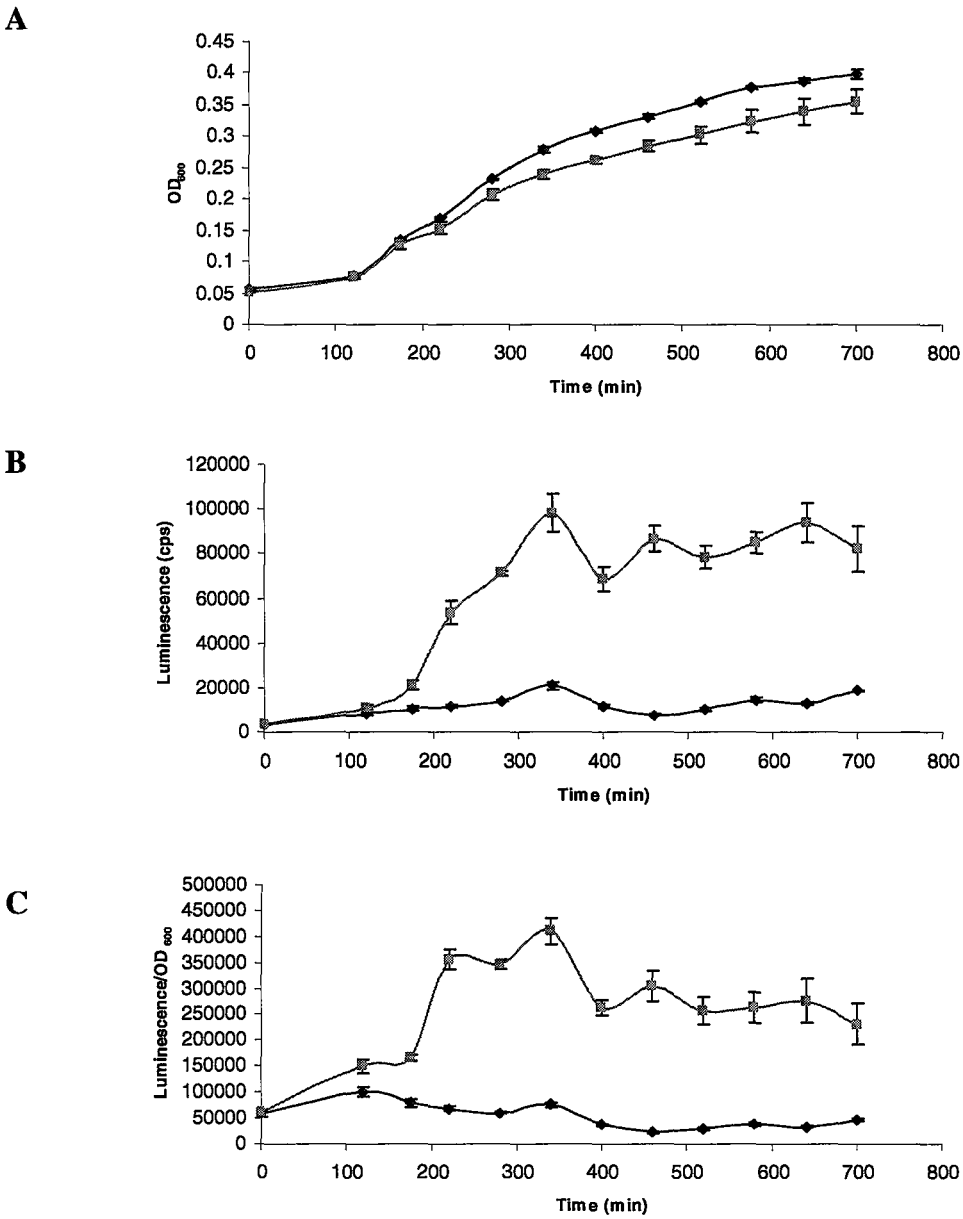
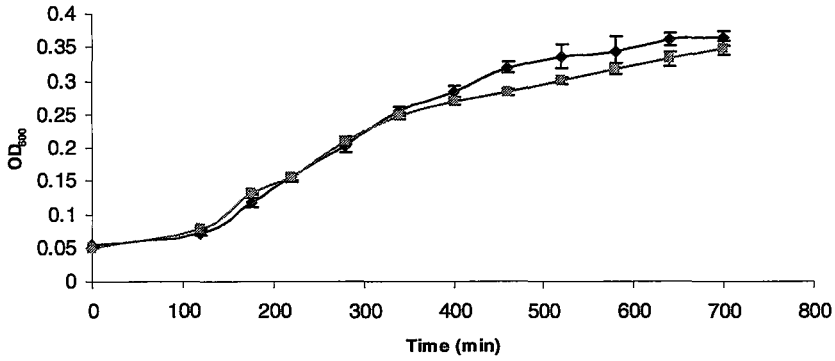
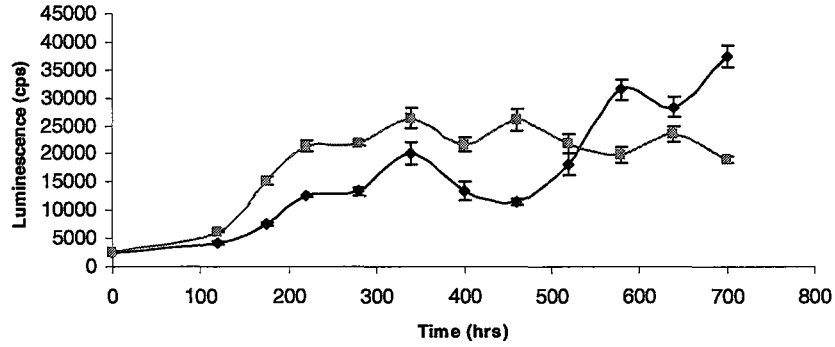


Figure 5.1-1. Effects of TagD-depletion on the P_{murB} reporter strain in the *tagD* conditional mutant background (EB1406). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (■) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

A



B



C

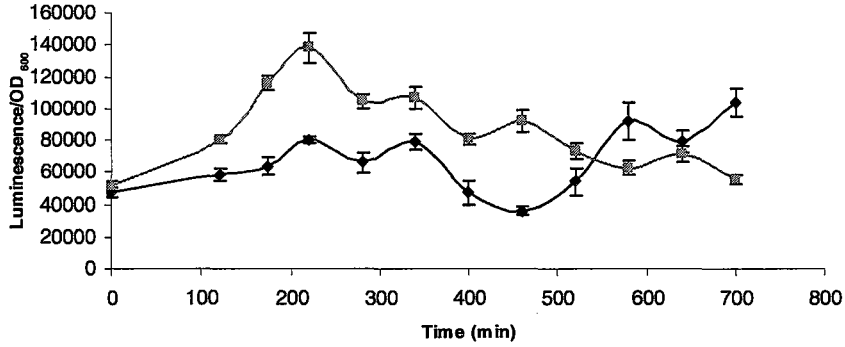


Figure 5.1-2. Effects of TagD-depletion on the P_{sers} reporter strain in the *tagD* conditional mutant background (EB1492). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (■) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

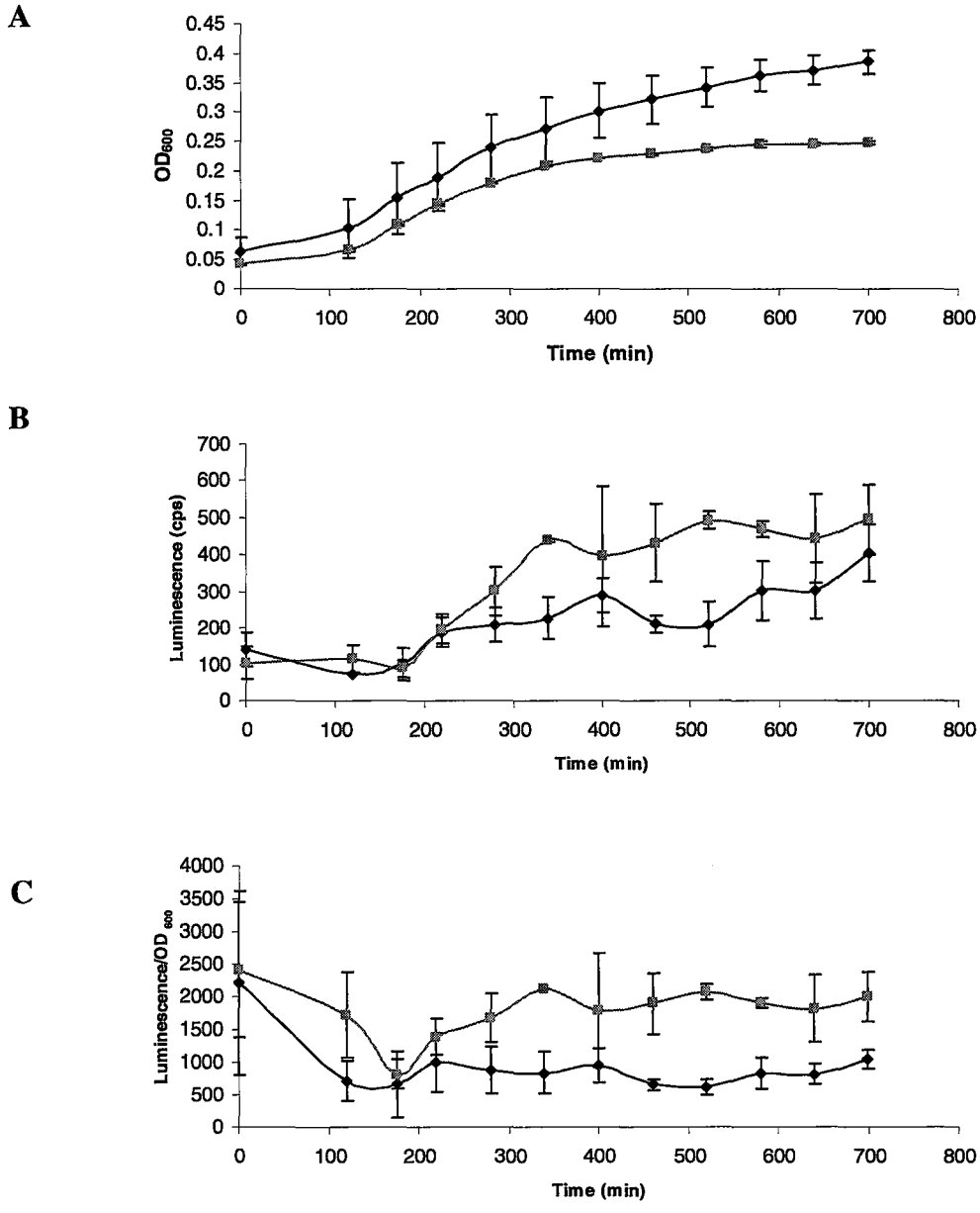


Figure 5.1-3. Effects of TagD-depletion on the P_{ydbO} reporter strain in the *tagD* conditional mutant background (EB1408). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

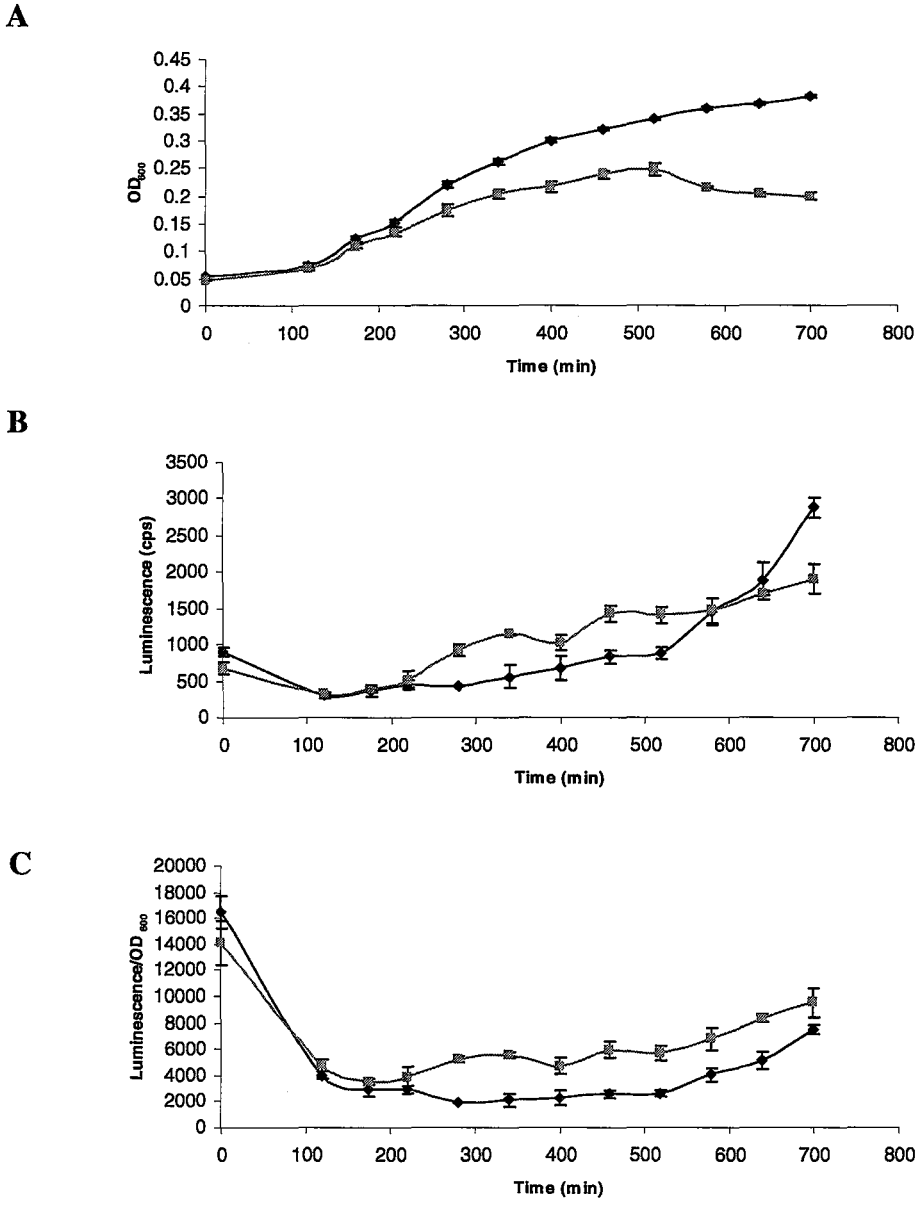
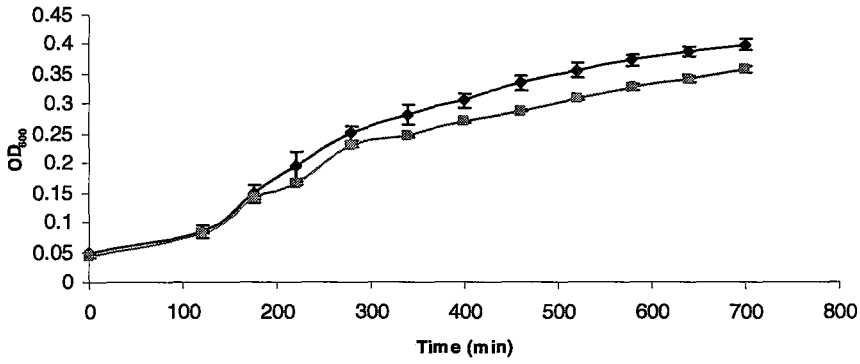
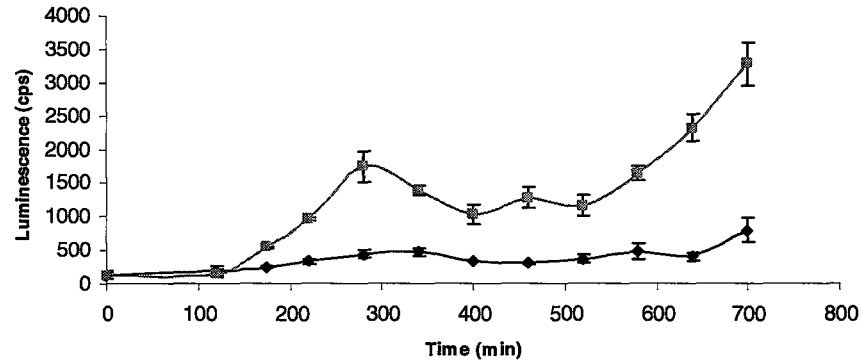


Figure 5.1-4. Effects of TagD-depletion on the P_{yfiB} reporter strain in the *tagD* conditional mutant background (EB1404). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (■) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

A



B



C

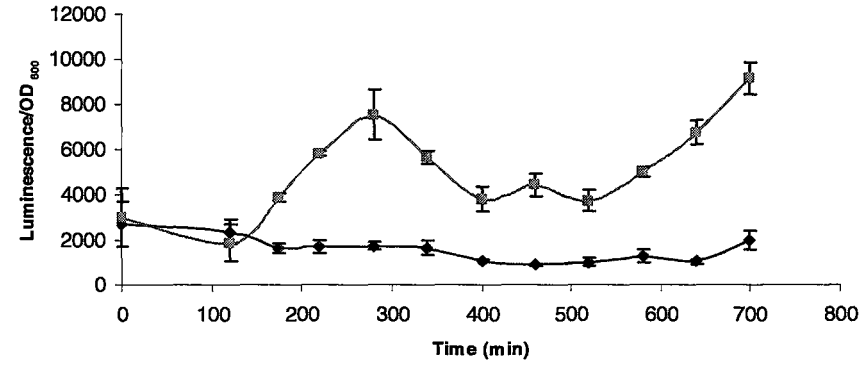


Figure 5.1-5. Effects of TagD-depletion on the P_{yhaU} reporter strain in the *tagD* conditional mutant background (EB1399). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (■) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

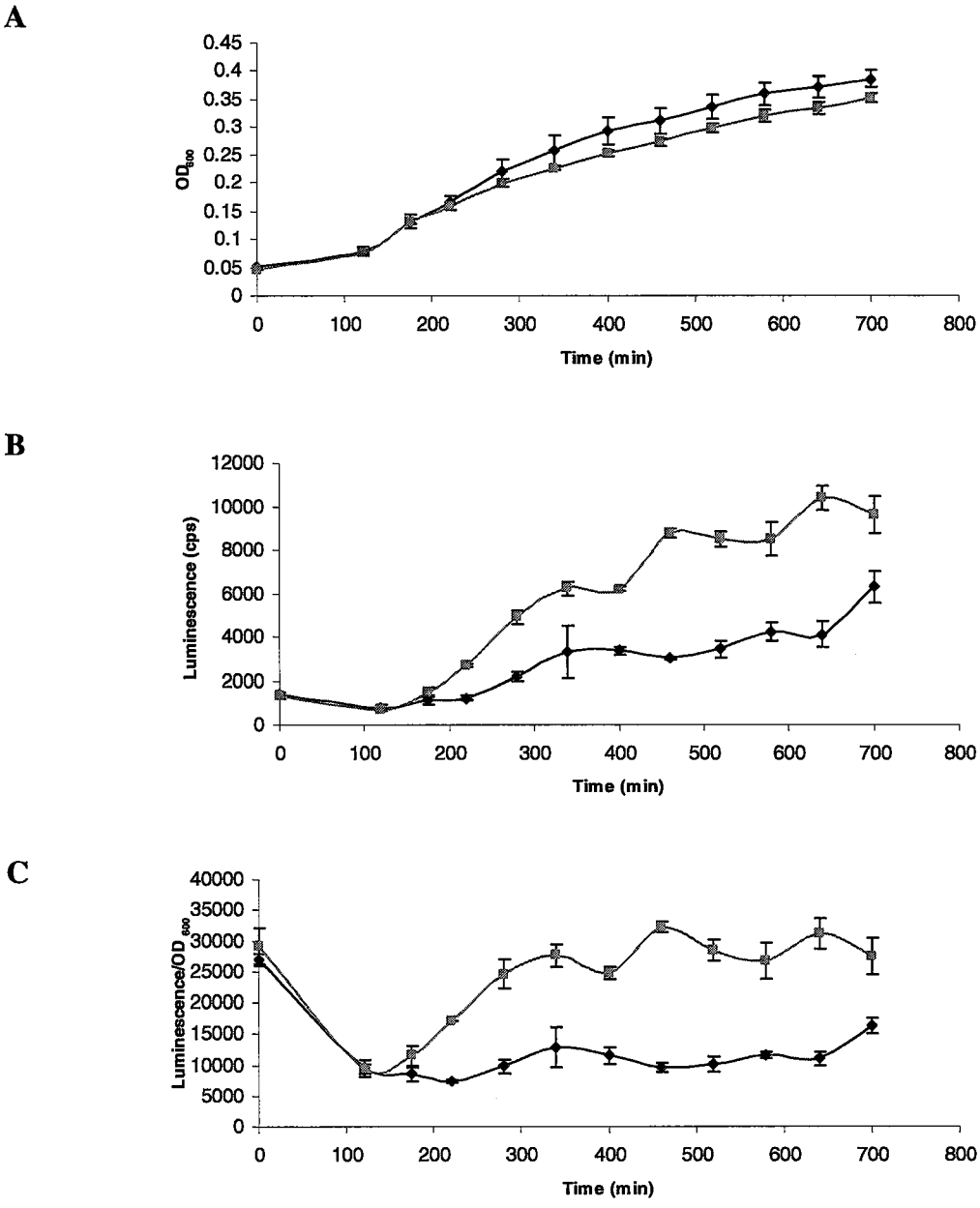


Figure 5.1-6. Effects of TagD-depletion on the P_{yktB} reporter strain in the *tagD* conditional mutant background (EB1400). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

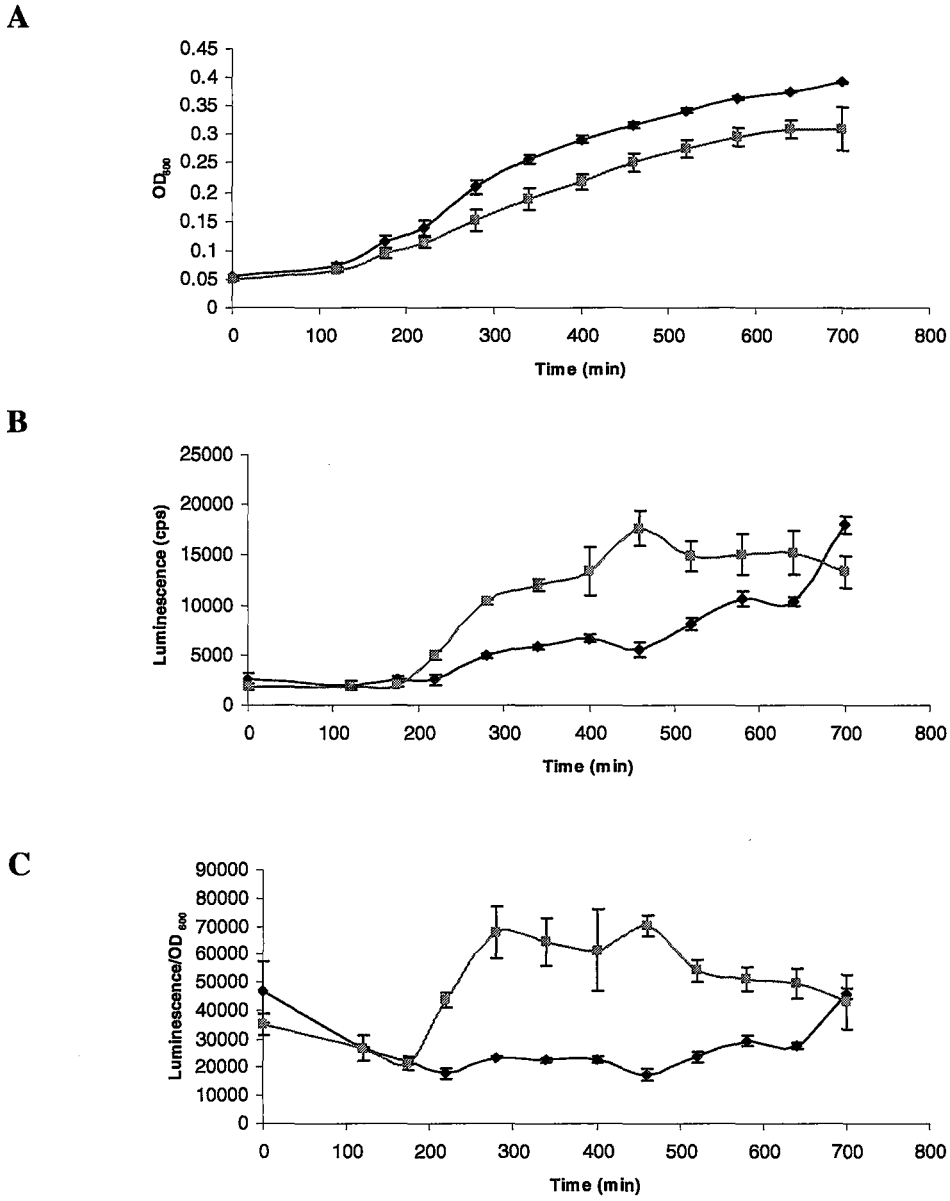
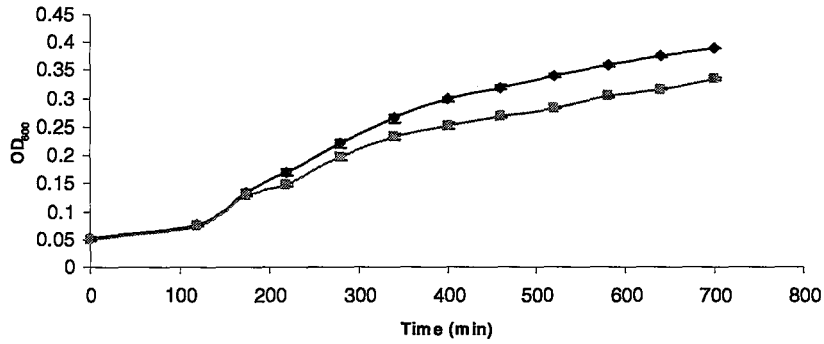
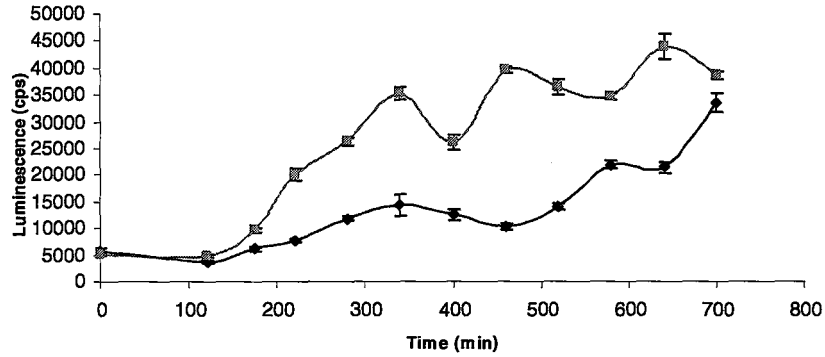


Figure 5.1-7. Effects of TagD-depletion on the P_{yqiW} reporter strain in the *tagD* conditional mutant background (EB1401). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

A



B



C

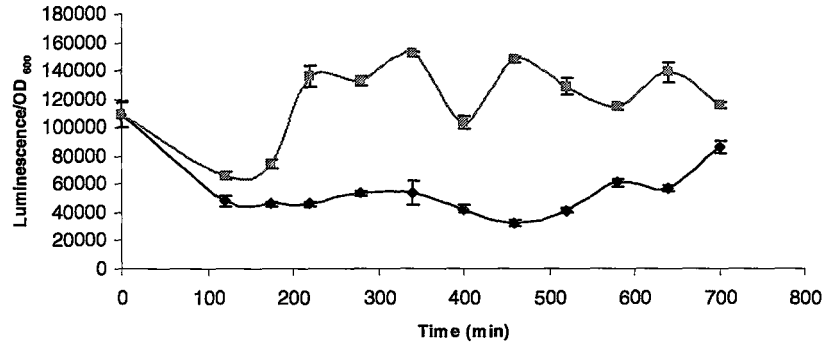
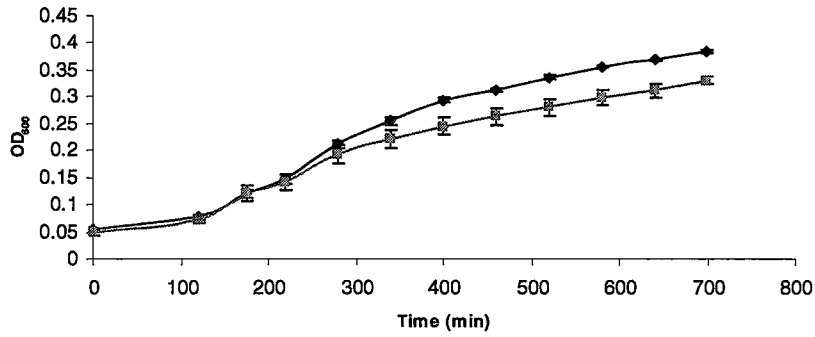
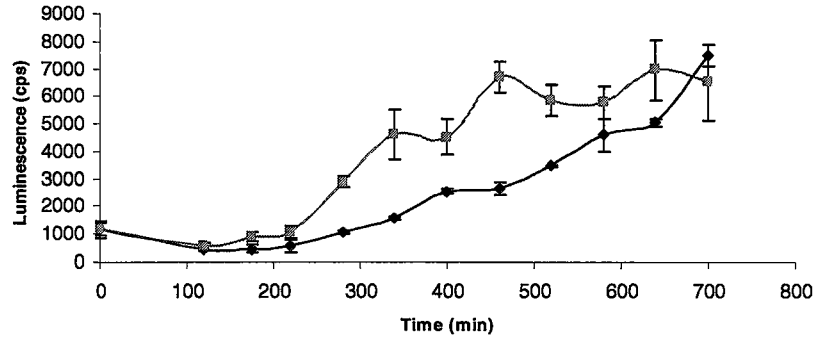


Figure 5.1-8. Effects of TagD-depletion on the P_{yubB} reporter strain in the *tagD* conditional mutant background (EB1407). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

A



B



C

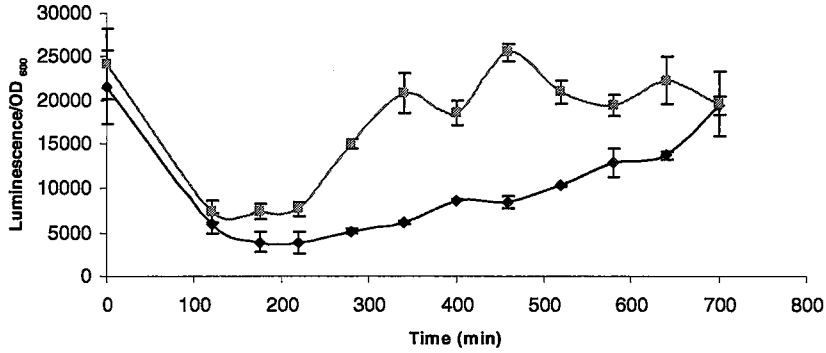


Figure 5.1-9. Effects of TagD-depletion on the P_{yvfT} reporter strain in the *tagD* conditional mutant background (EB1403). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was

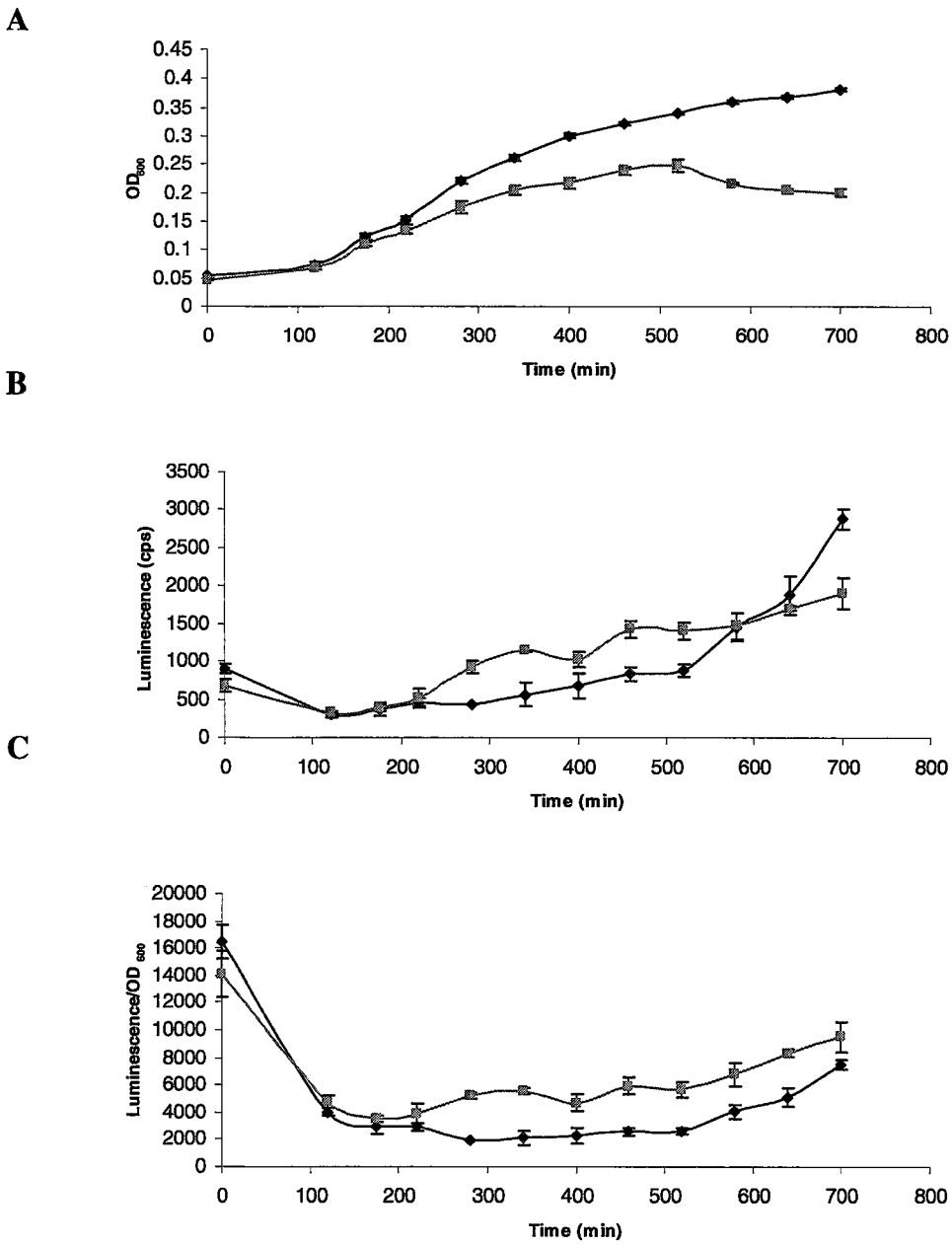


Figure 5.1-10. Effects of TagD-depletion on the P_{yxel} reporter strain in the *tagD* conditional mutant background (EB1405). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

Appendix 5.2: Response of reporter strains to TagB-depletion

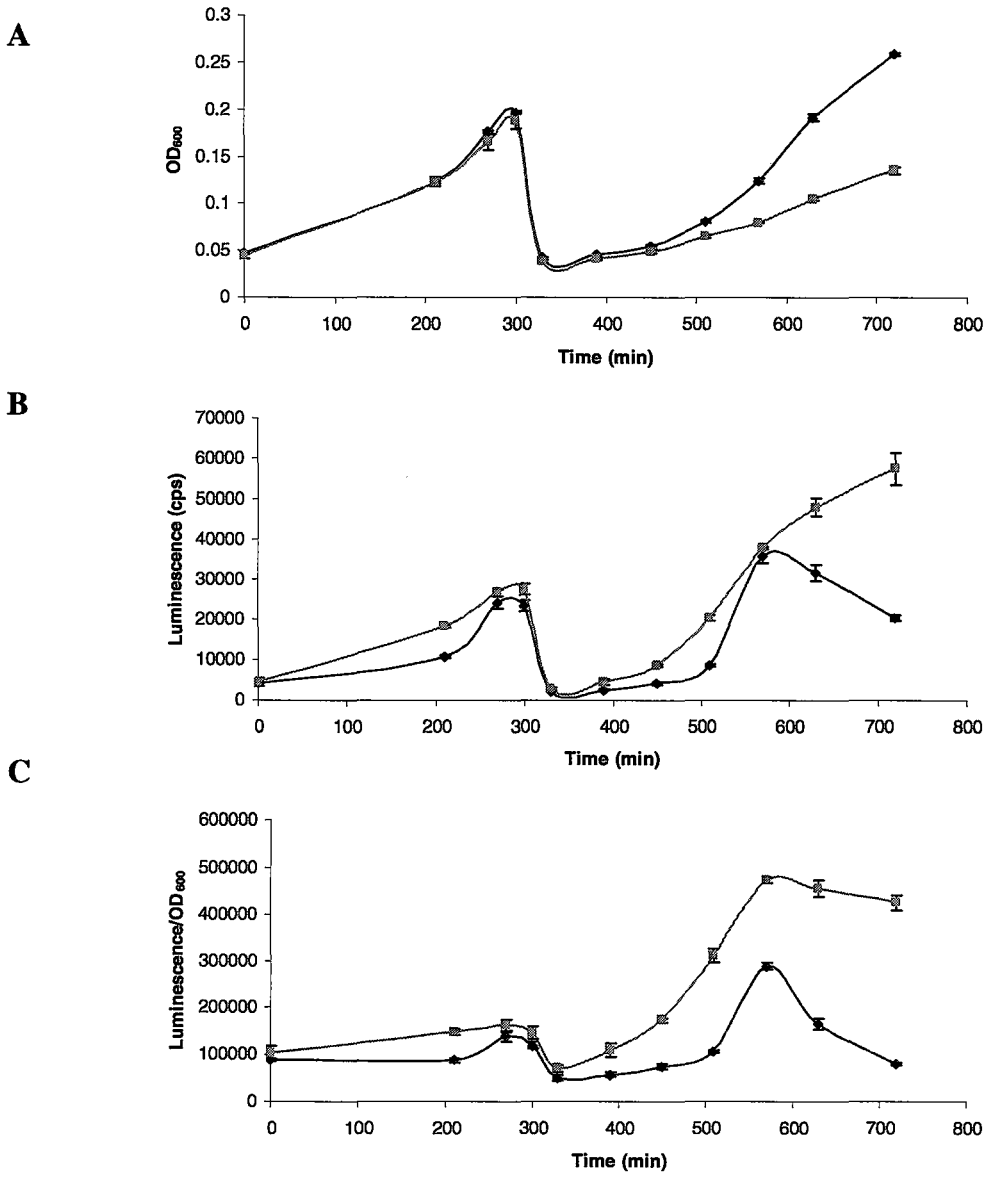


Figure 5.2-1. Effects of TagB-depletion on the P_{murB} reporter strain in the *tagB* conditional mutant background (EB1552). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

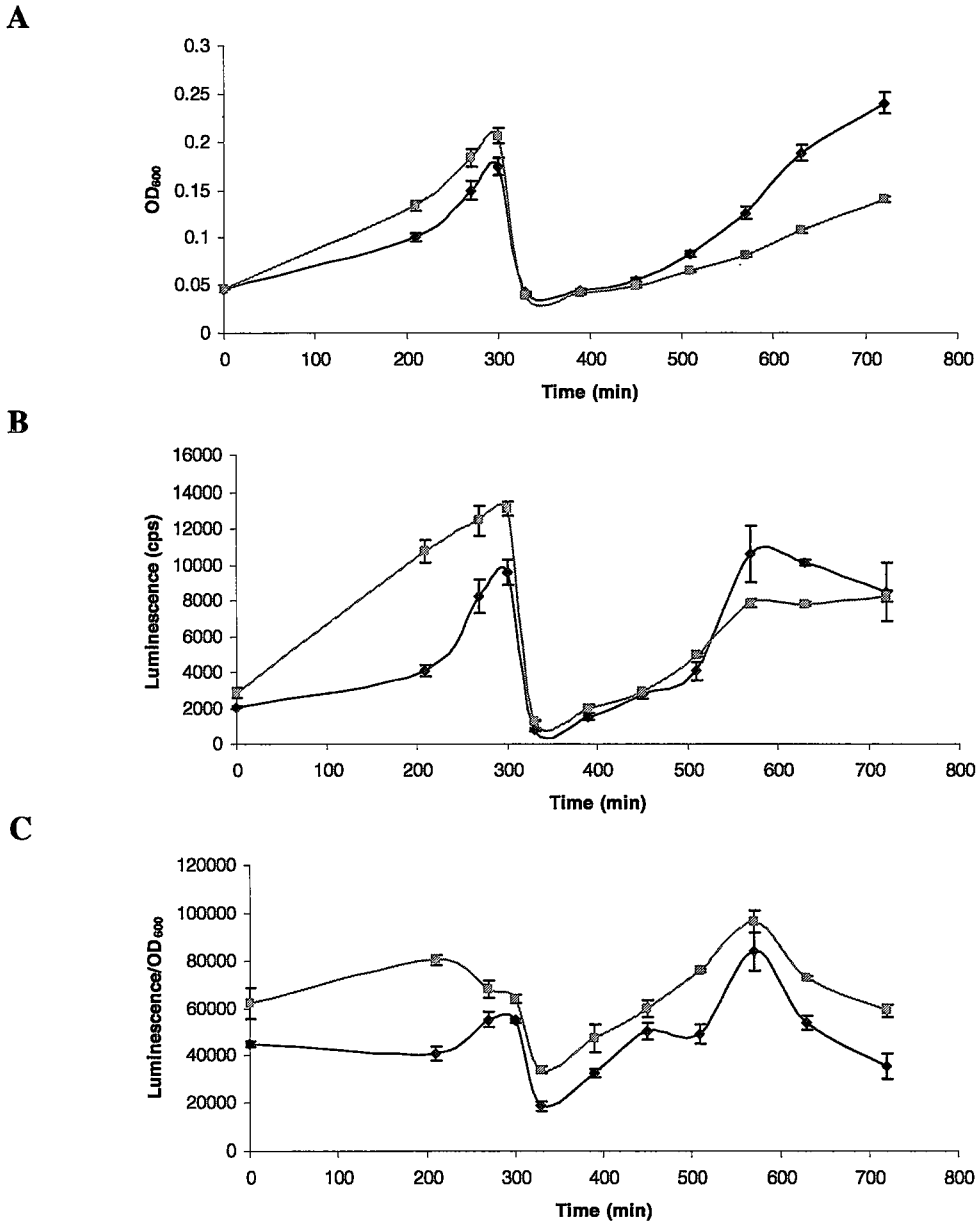
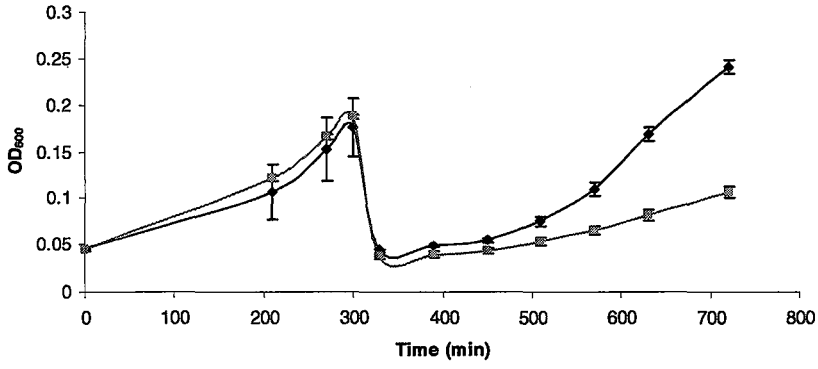
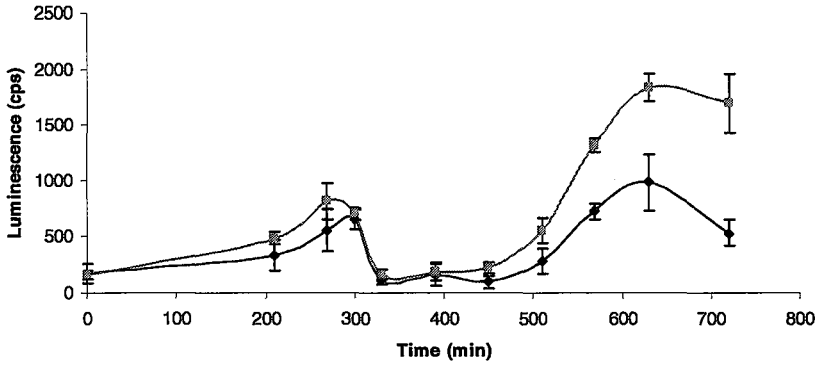


Figure 5.2-2. Effects of TagB-depletion on the P_{sers} reporter strain in the *tagB* conditional mutant background (EB1554). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▨) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

A



B



C

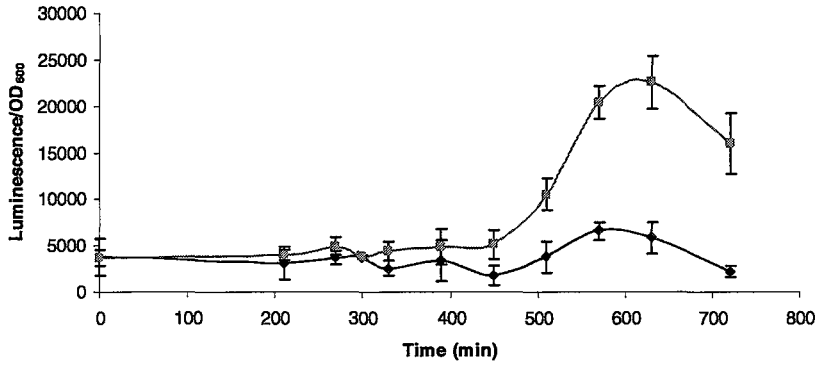


Figure 5.2-3. Effects of TagB-depletion on the P_{yhaU} reporter strain in the *tagB* conditional mutant background (EB1553). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

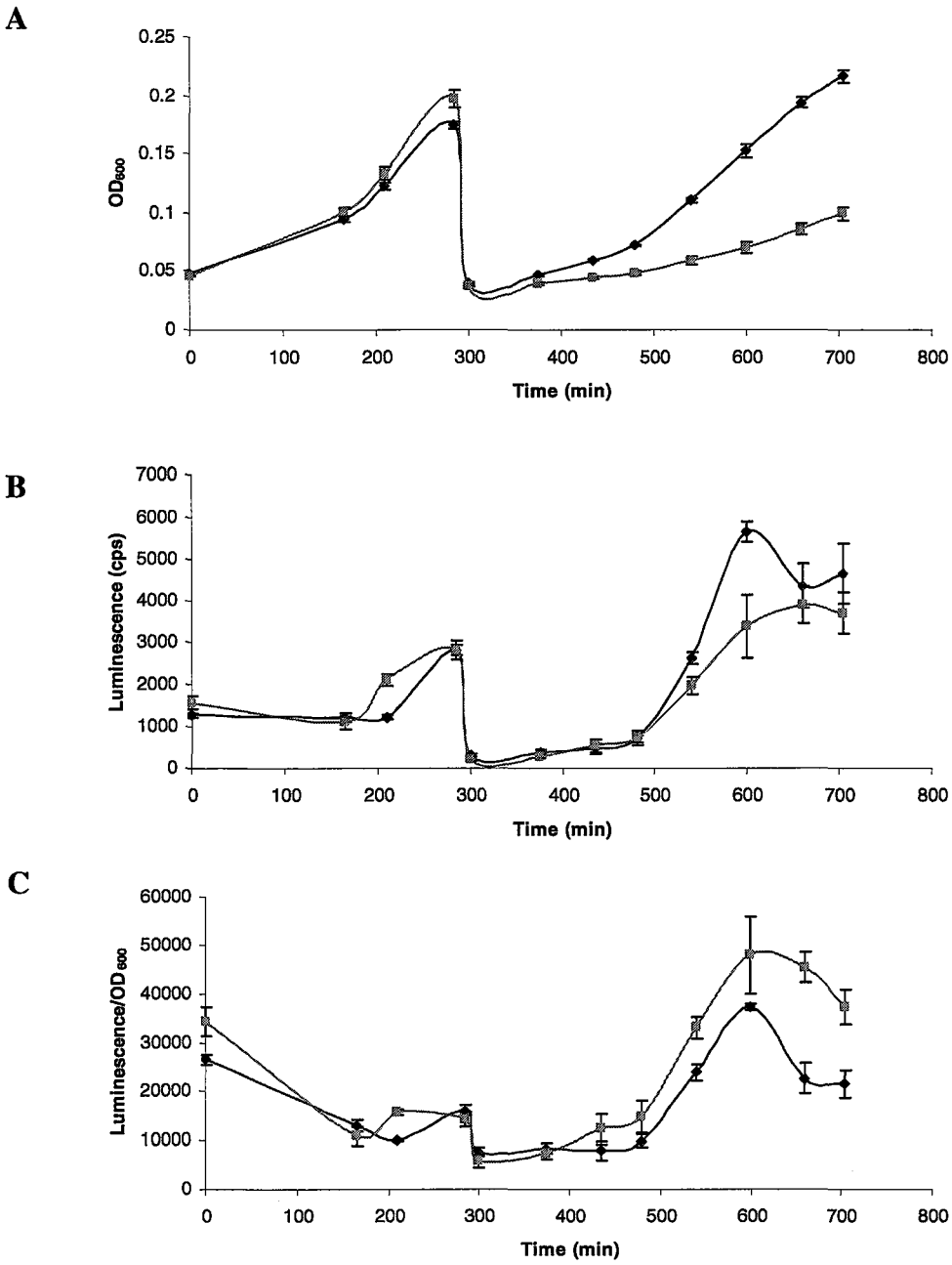


Figure 5.2-4. Effects of TagB-depletion on the P_{yktB} reporter strain in the *tagB* conditional mutant background (EB1550). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (■) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

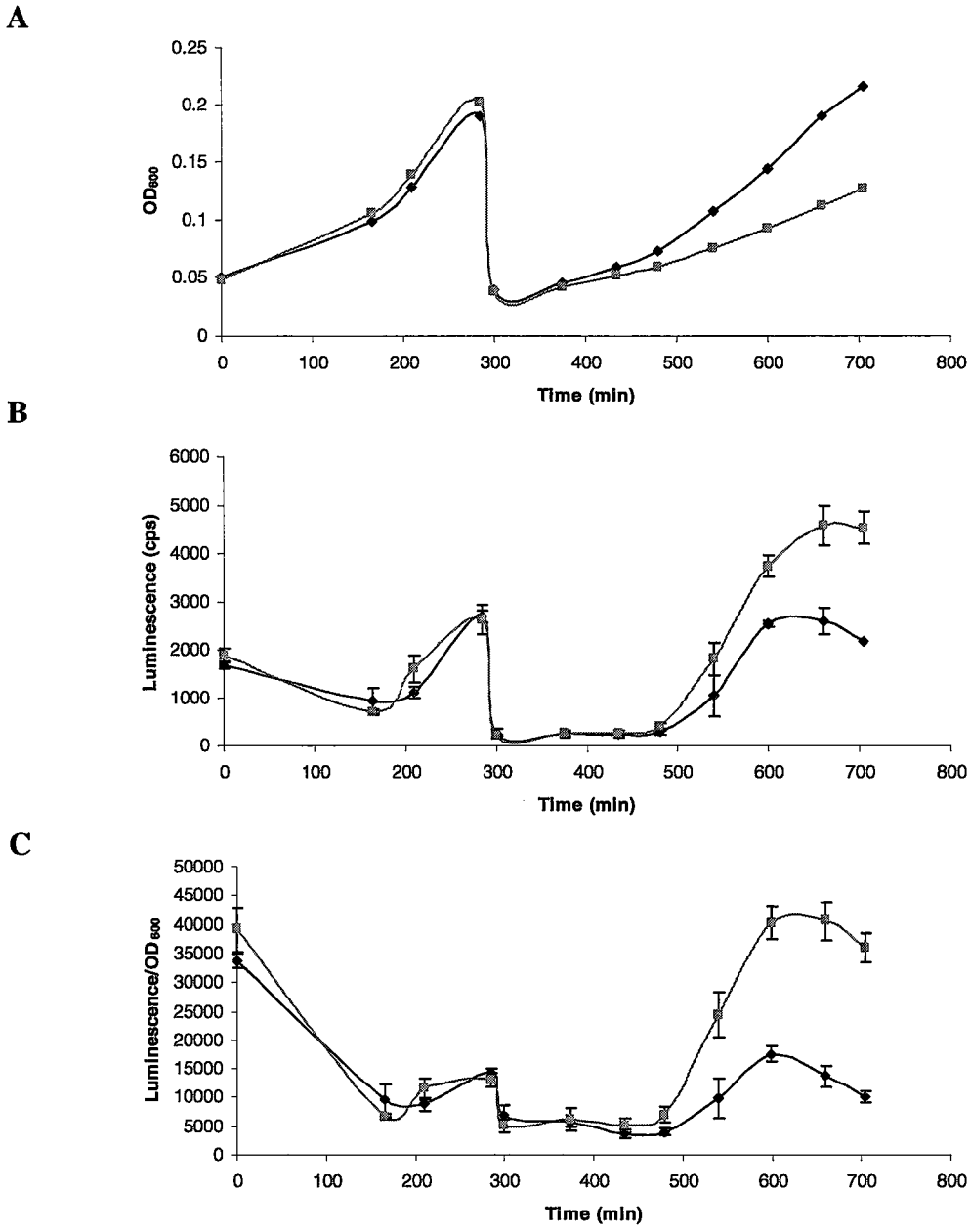
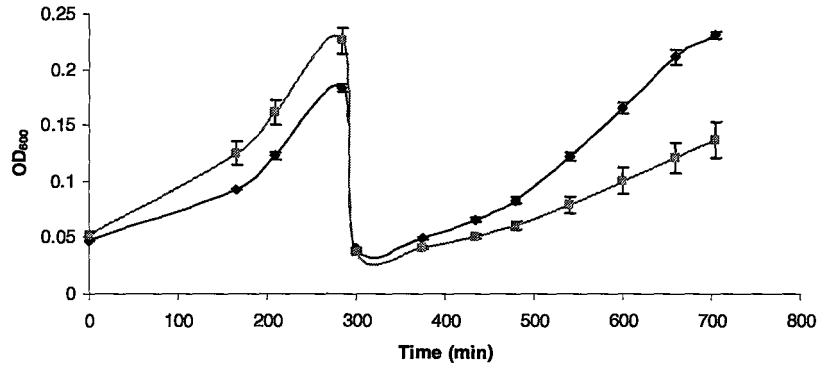
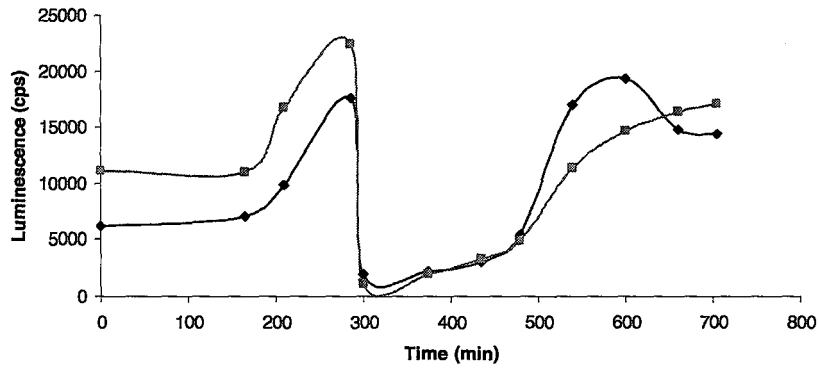


Figure 5.2-5. Effects of TagB-depletion on the P_{yubB} reporter strain in the *tagB* conditional mutant background (EB1485). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (■) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). T A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

A



B



C

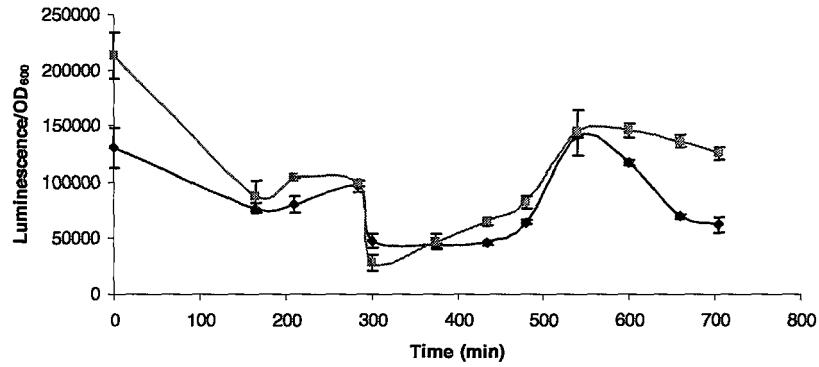
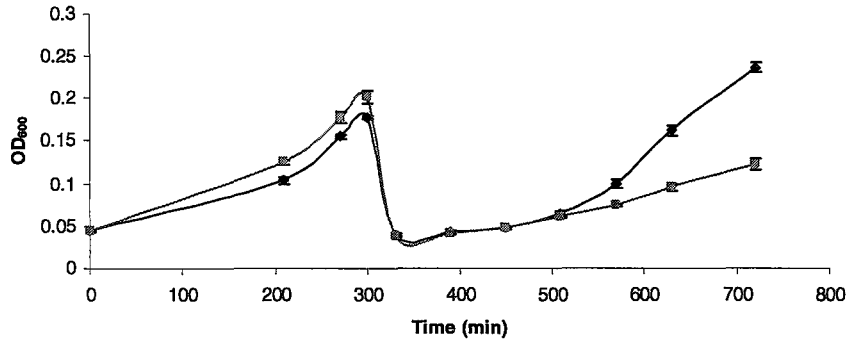
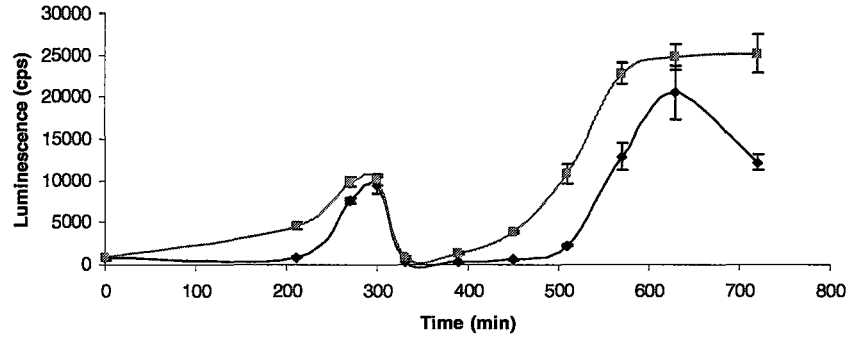


Figure 5.2-6. Effects of TagB-depletion on the P_{yvfT} reporter strain in the *tagB* conditional mutant background (EB1555). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

A



B



C

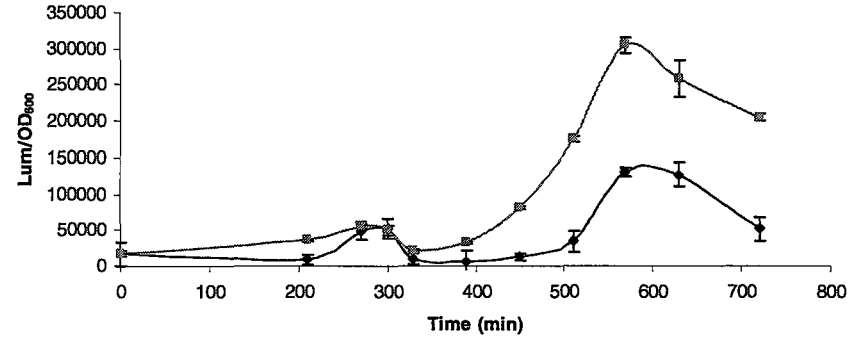


Figure 5.2-7. Effects of TagB-depletion on the P_{ywaC} reporter strain in the *tagB* conditional mutant background (EB1551). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

Appendix 5.3: Response of reporter strains to TagF-depletion

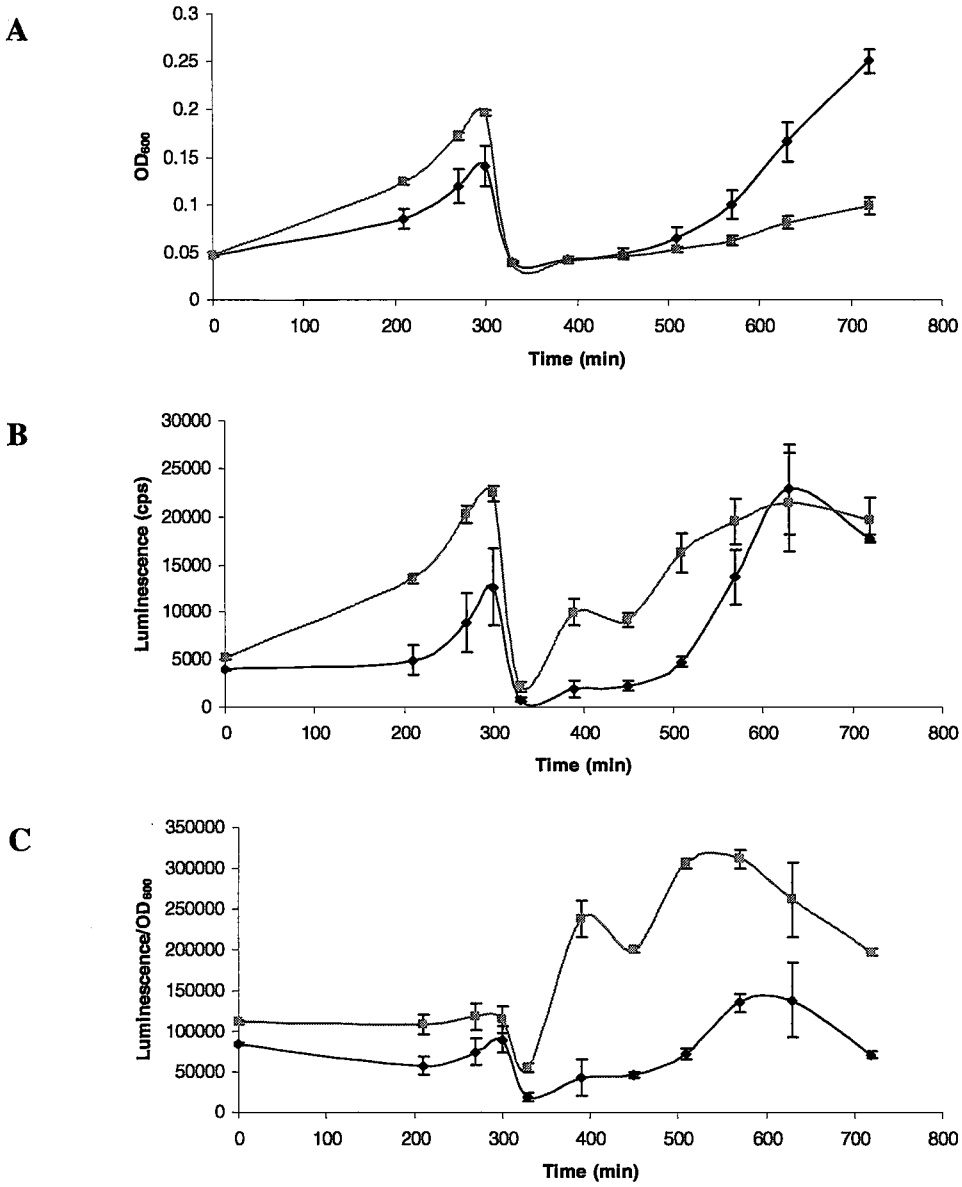


Figure 5.3-1. Effects of TagF-depletion on the P_{murB} reporter strain in the *tagF* conditional mutant background (EB1434). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

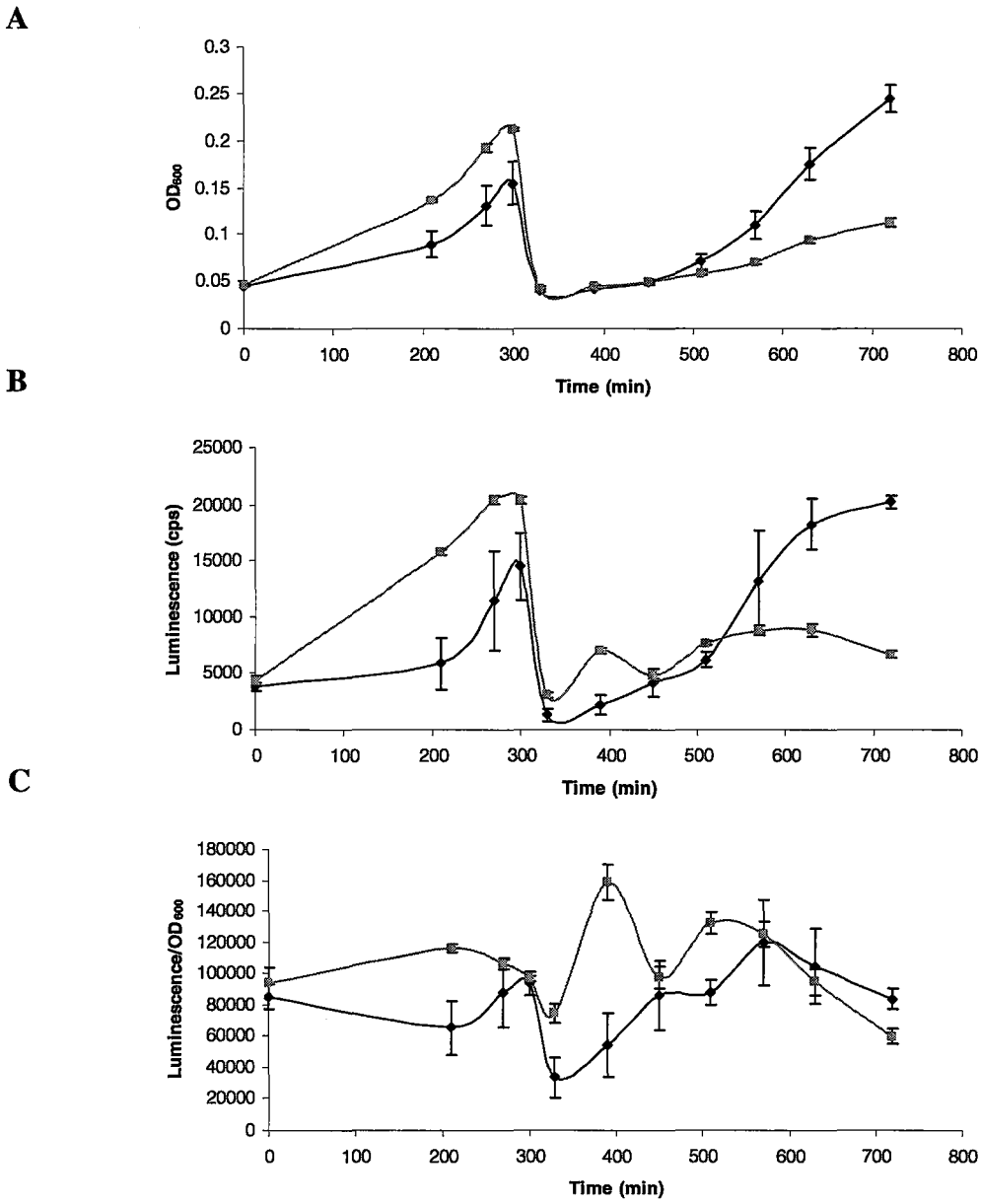


Figure 5.3-2. Effects of TagF-depletion on the P_{serS} reporter strain in the *tagF* conditional mutant background (EB1549). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

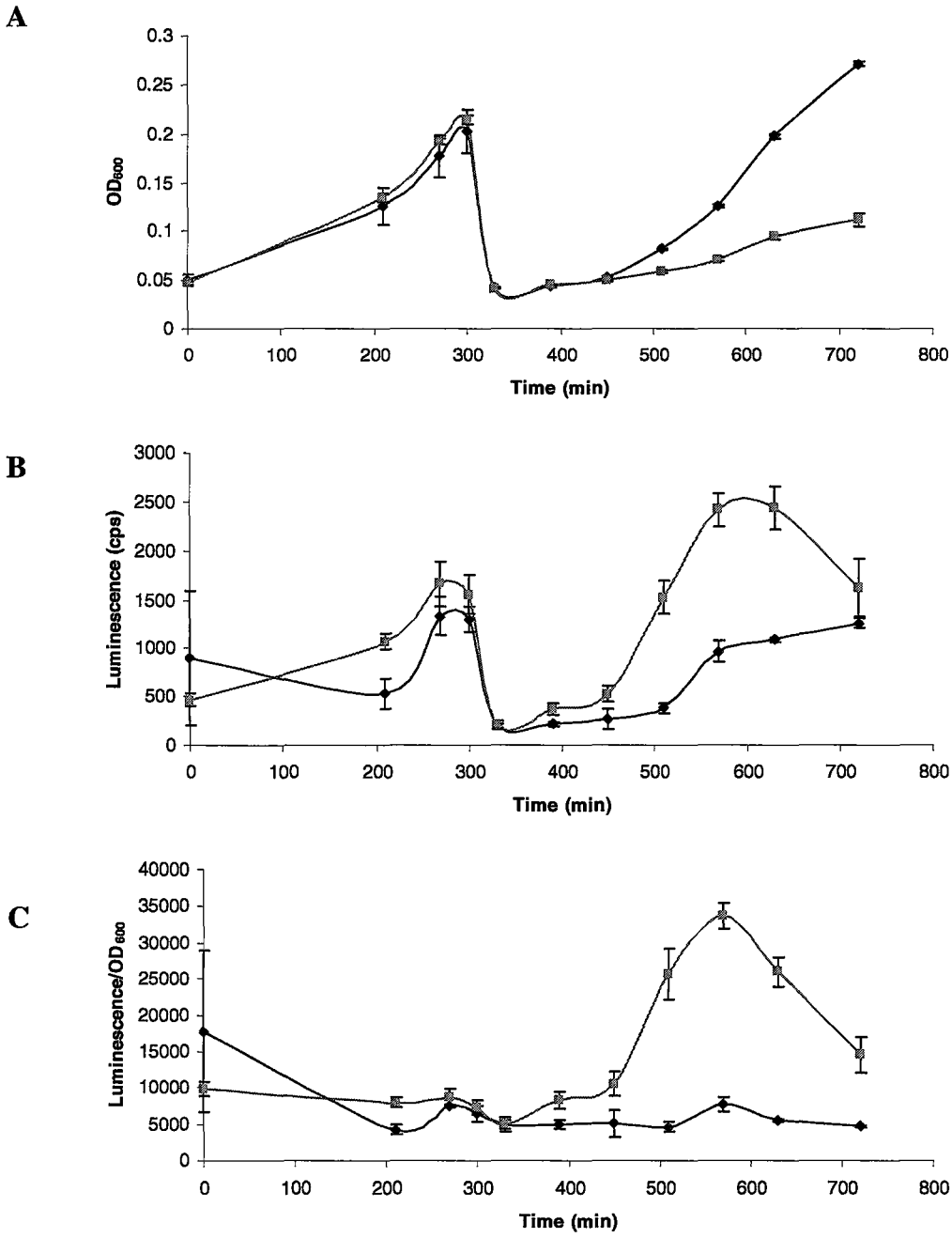


Figure 5.3-3. Effects of TagF-depletion on the P_{yhaU} reporter strain in the *tagF* conditional mutant background (EB1548). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (■) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

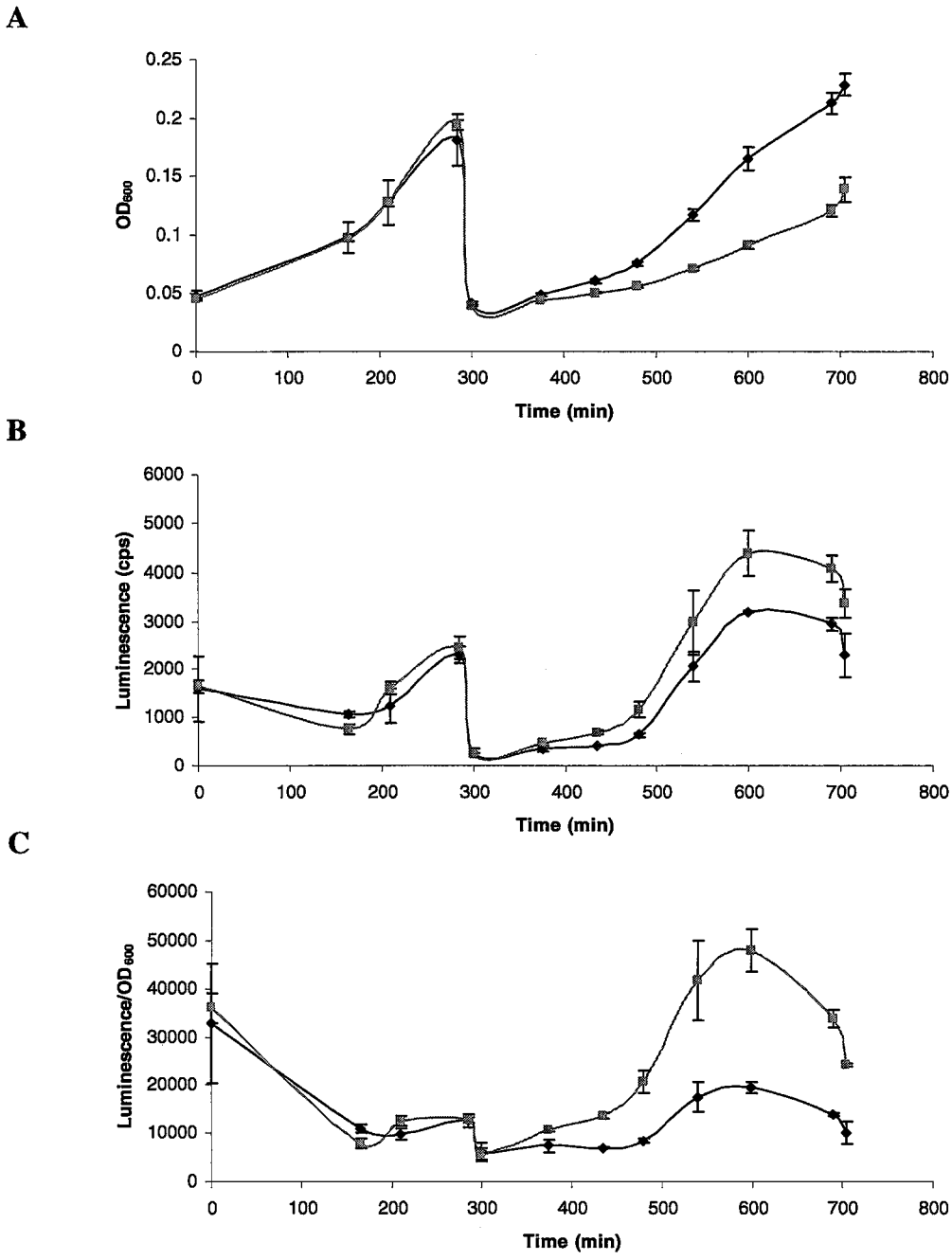


Figure 5.3-4. Effects of TagF-depletion on the P_{ykzB} reporter strain in the *tagF* conditional mutant background (EB1433). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

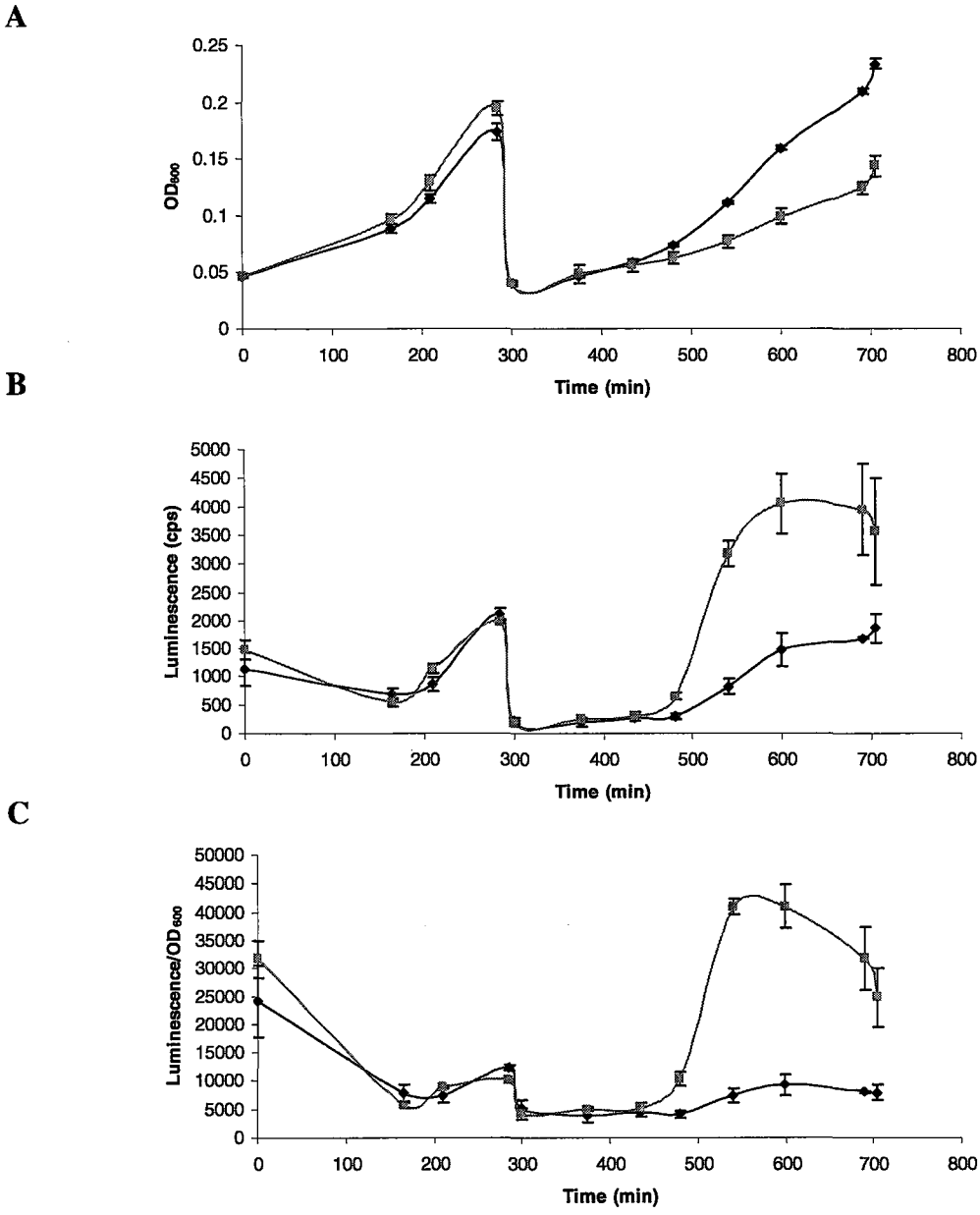


Figure 5.3-5. Effects of TagF-depletion on the P_{yubB} reporter strain in the *tagF* conditional mutant background (EB1432). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (■) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

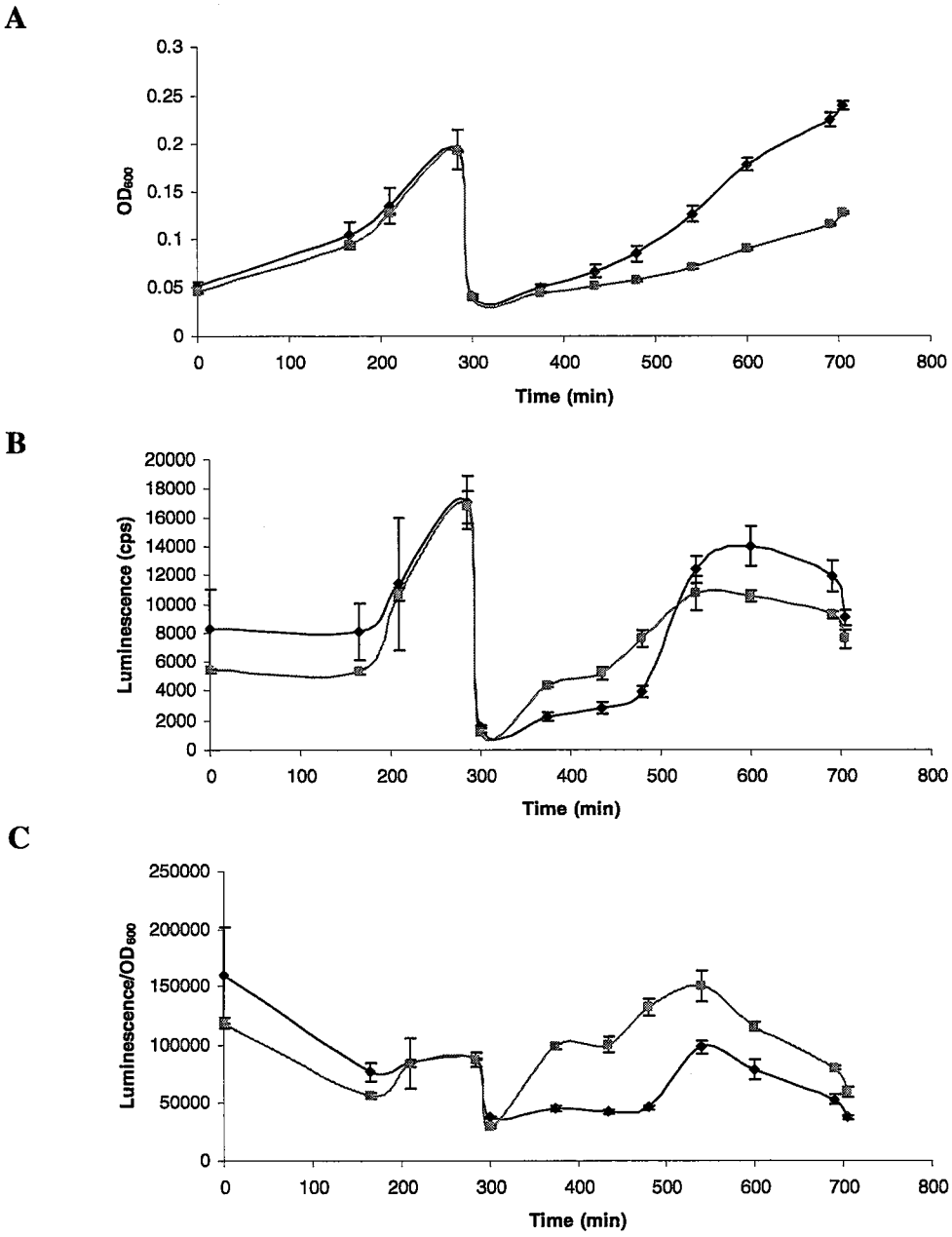


Figure 5.3-6. Effects of TagF-depletion on the P_{yvfT} reporter strain in the *tagF* conditional mutant background (EB1547). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

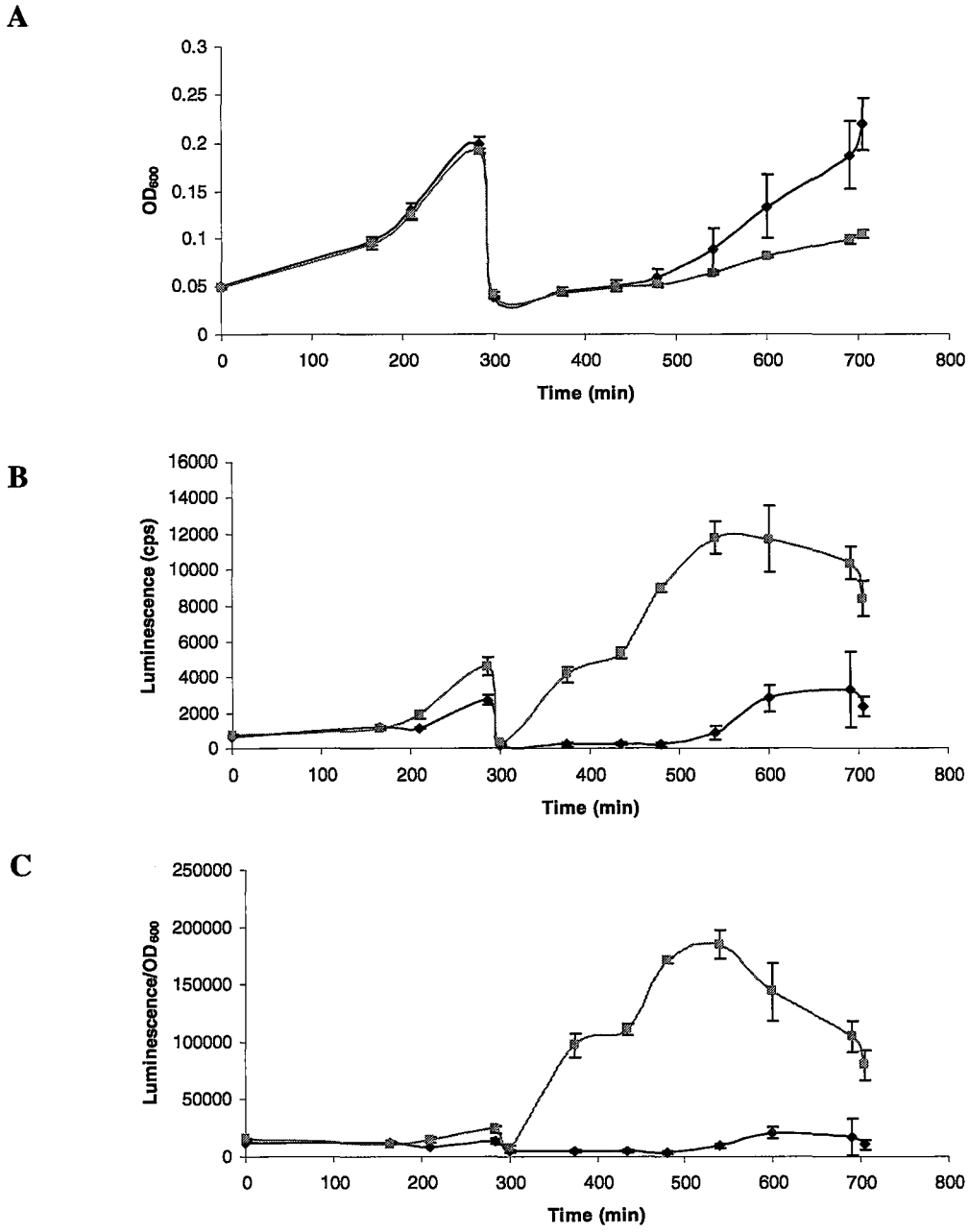


Figure 5.3-7. Effects of TagF-depletion on the P_{ywaC} reporter strain in the *tagF* conditional mutant background (EB1546). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (■) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). A second 100-fold dilution was made into fresh media in the presence and absence of xylose after 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

Appendix 5.4: Response of reporter strains to YacN-depletion

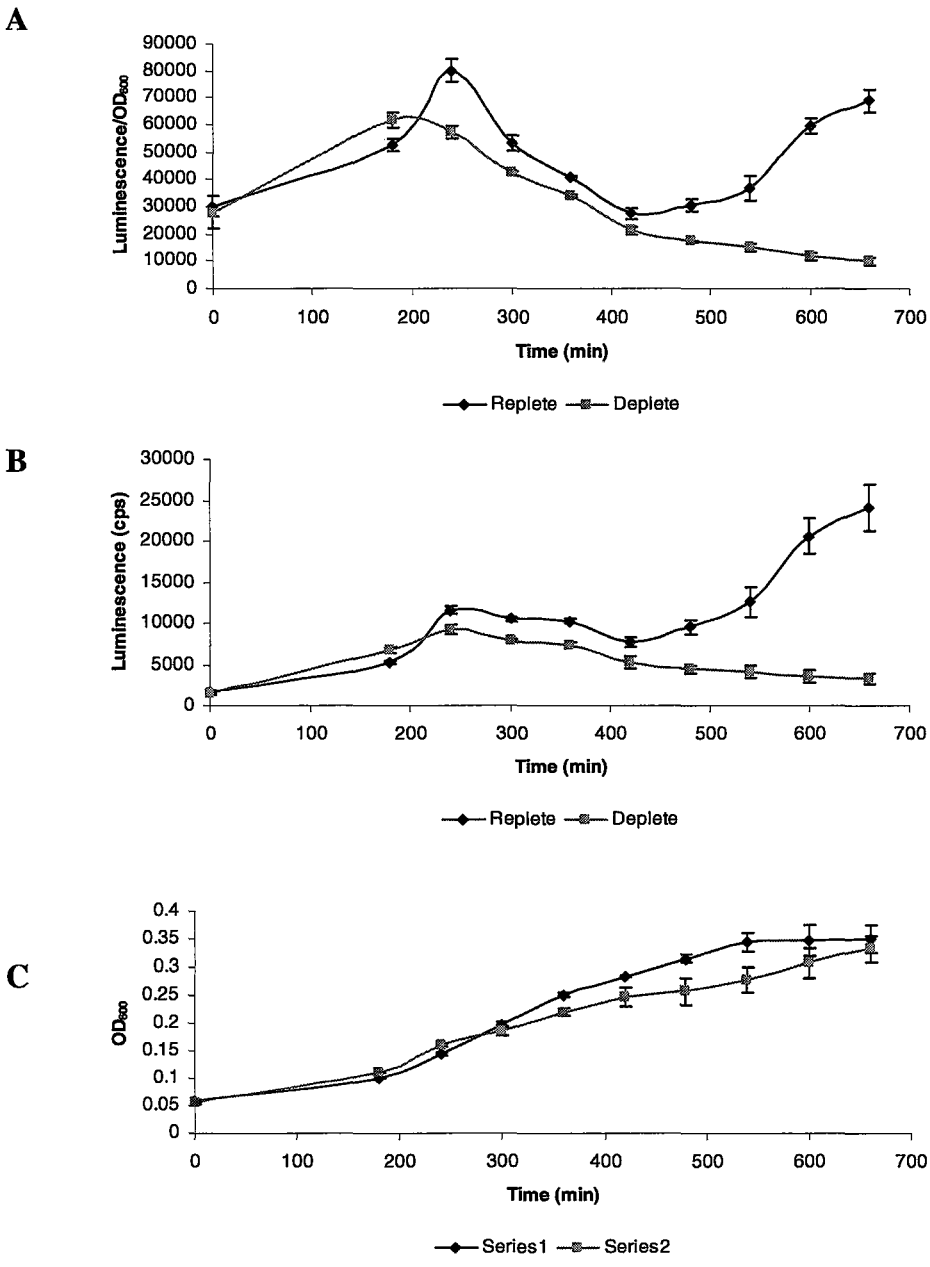
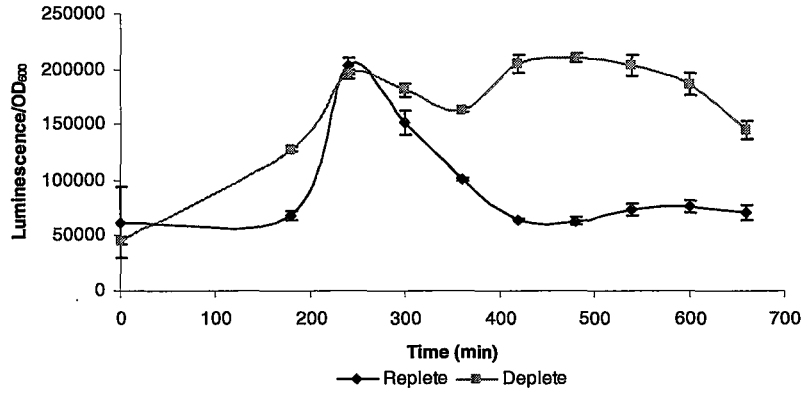
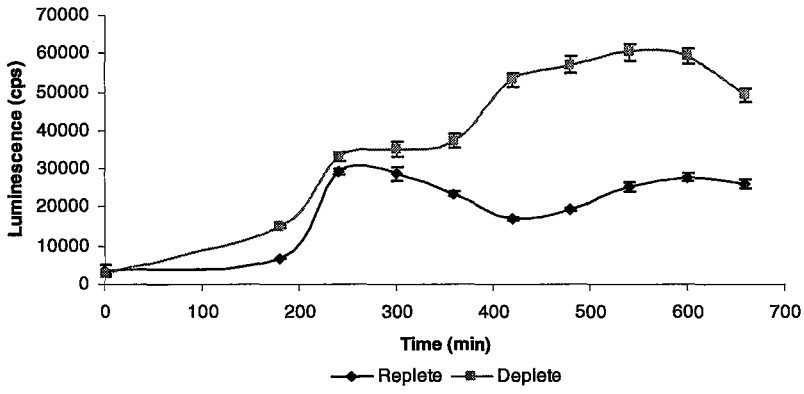


Figure 5.4-1. Effects of YacN-depletion on the P_{serS} reporter strain in the *yacN* conditional mutant background (EB1594). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (▩) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

A



B



C

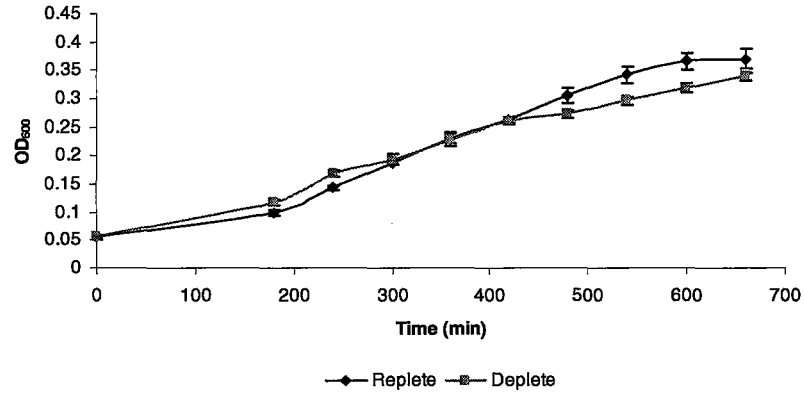


Figure 5.4-2. Effects of YacN-depletion on the P_{ywaC} reporter strain in the *yacN* conditional mutant background (EB1593). This strain was grown in LB supplemented with erythromycin and spectinomycin in the absence (■) and presence (◆) of the inducer (2 % xylose). Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

Appendix 5.5: Response of P_{ywaC} to various antibiotics

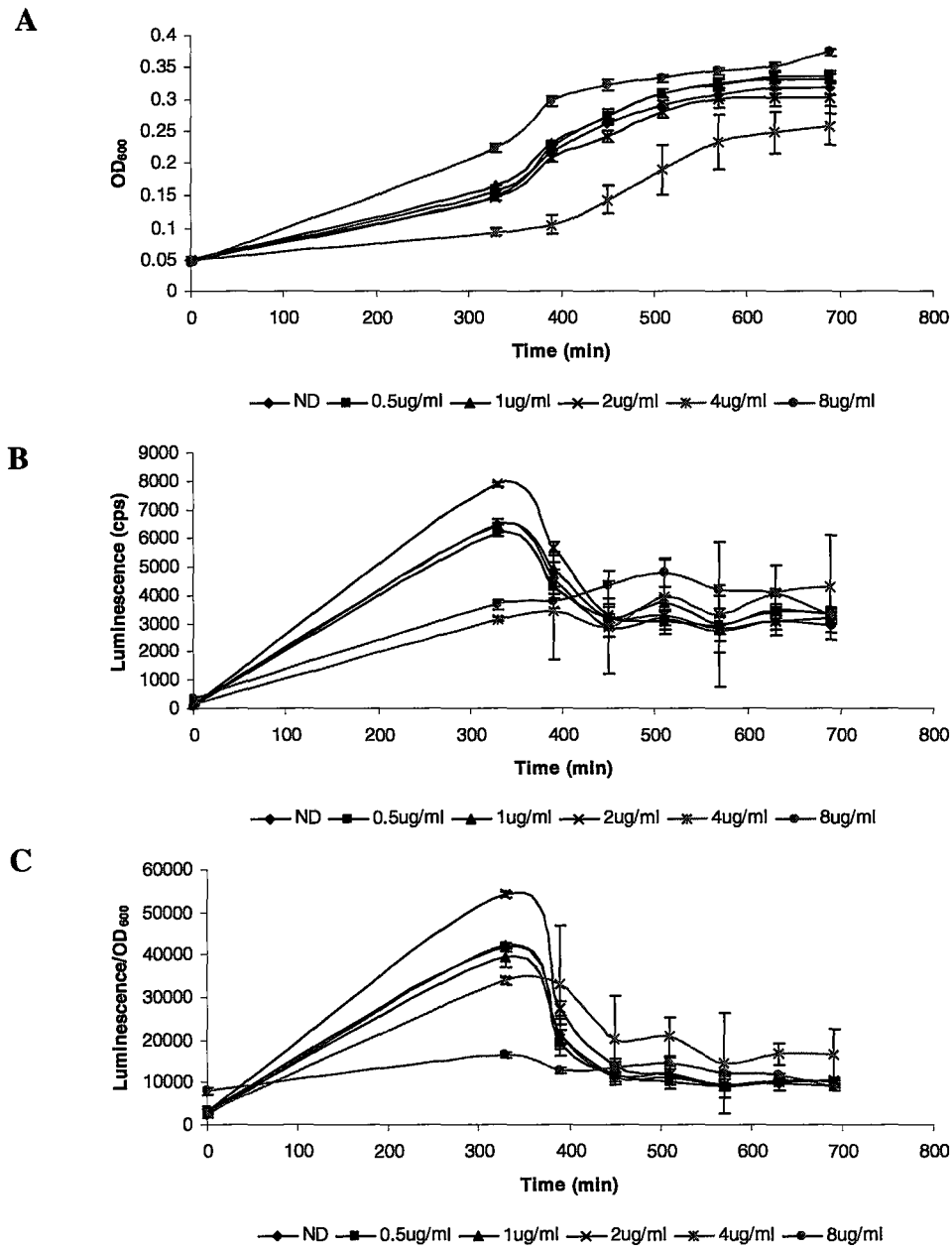


Figure 5.5-1. Effect of ampicillin on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Ampicillin was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

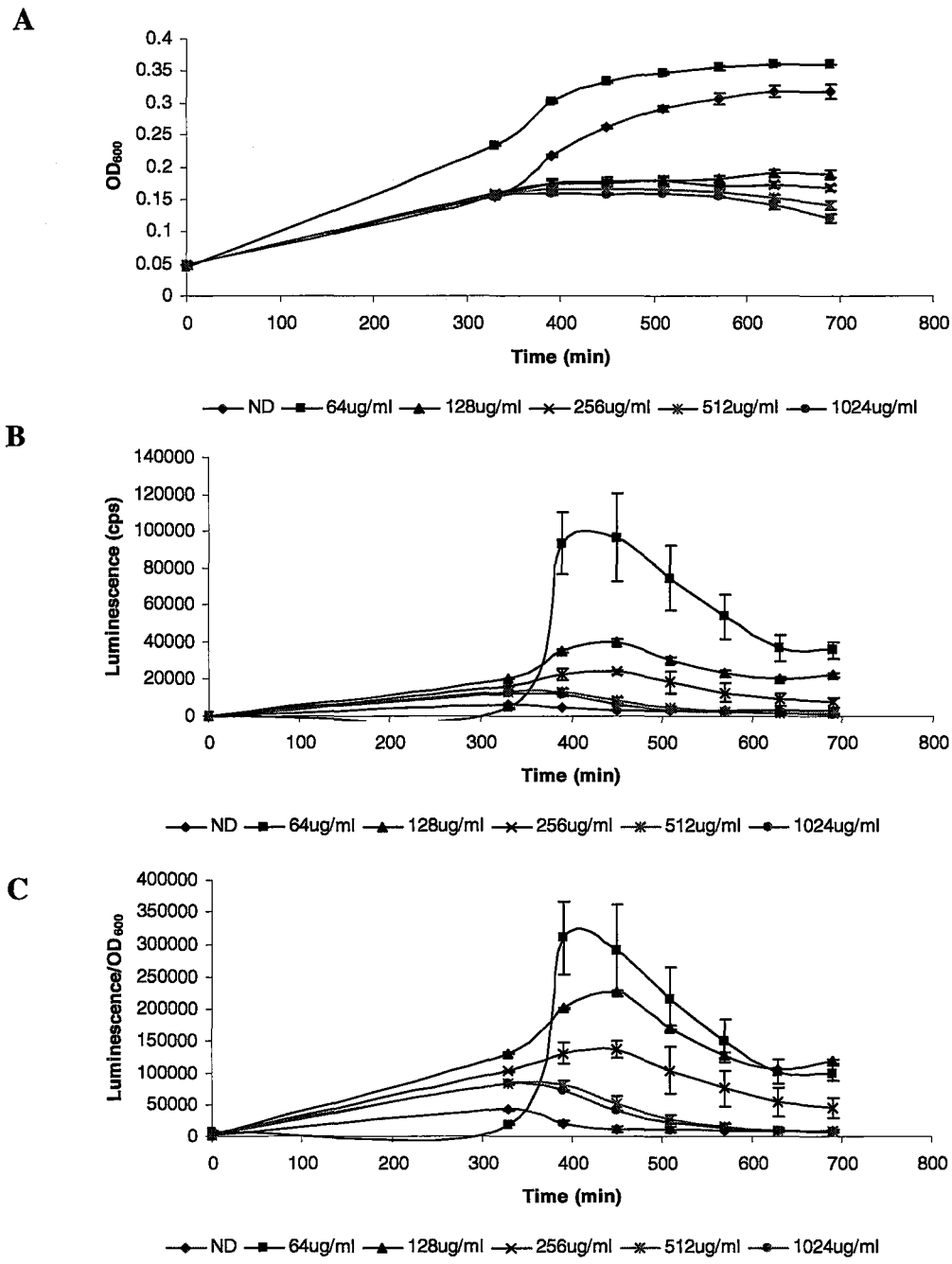


Figure 5.5-2. Effect of bacitracin on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Bacitracin was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

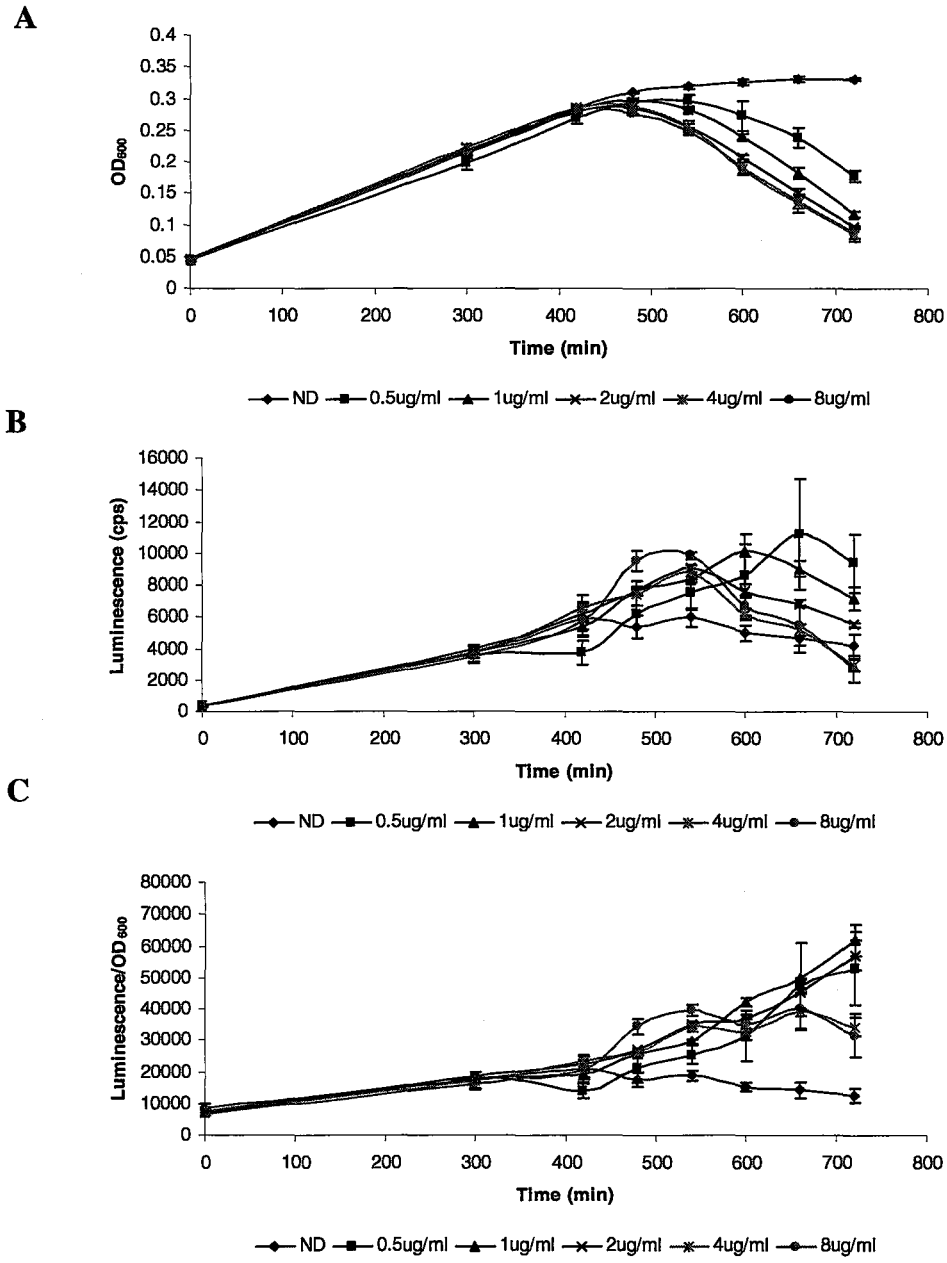


Figure 5.5-3. Effect of cefotaxime on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Cefotaxime was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

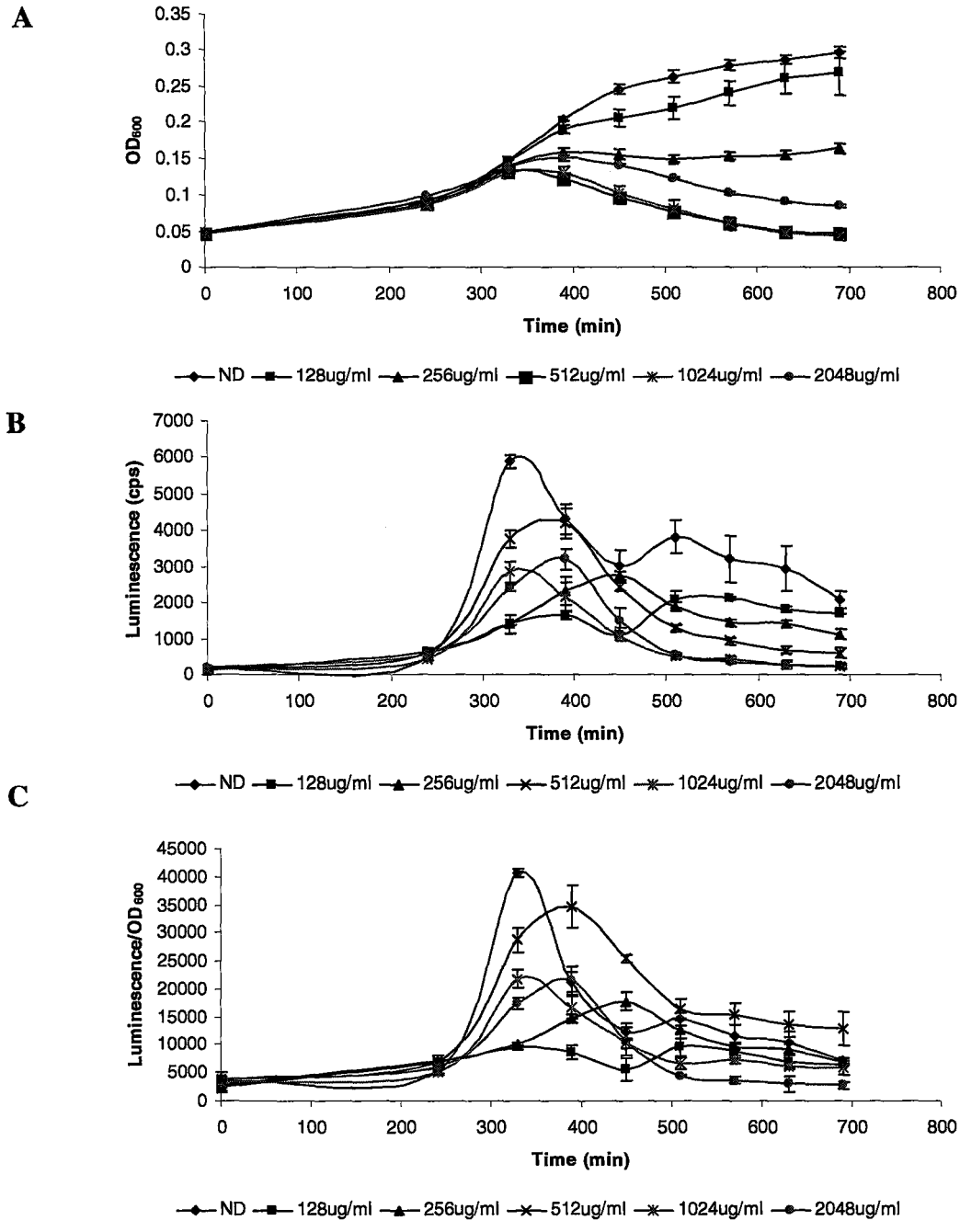


Figure 5.5-4. Effect of cycloserine on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Cycloserine was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

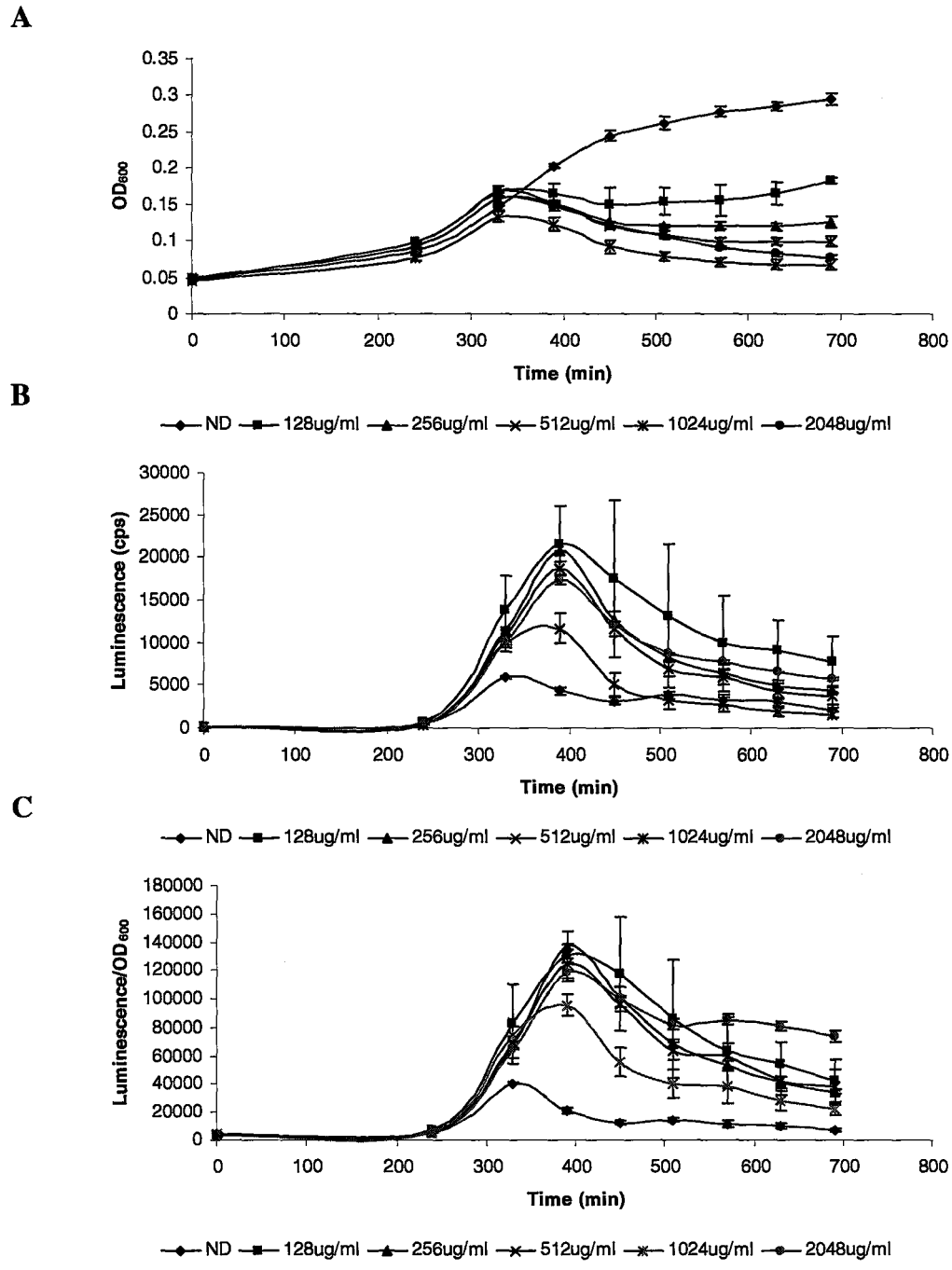


Figure 5.5-5. Effect of fosfomycin on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Fosfomycin was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

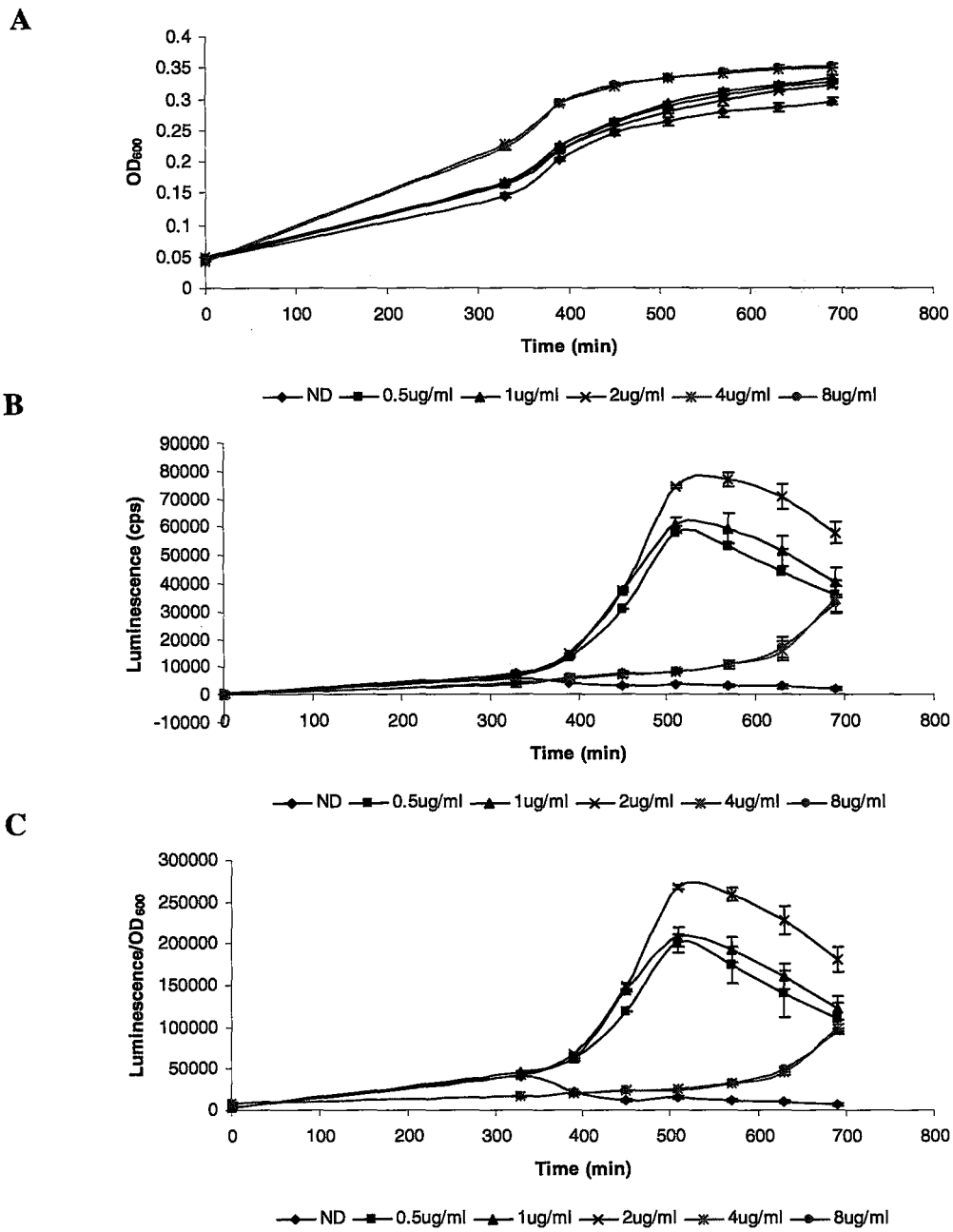
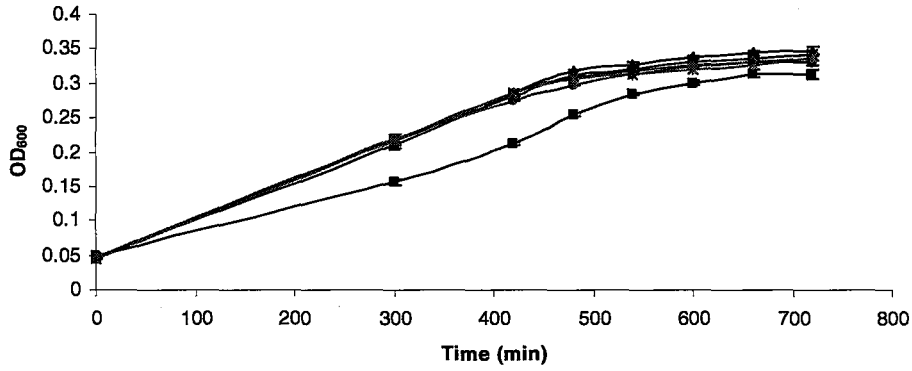
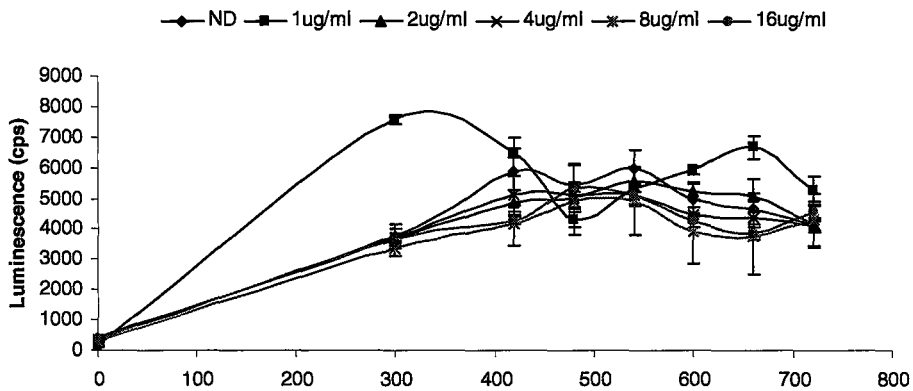


Figure 5.5-6. Effect of fosmidomycin on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Fosmidomycin was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

A



B



C

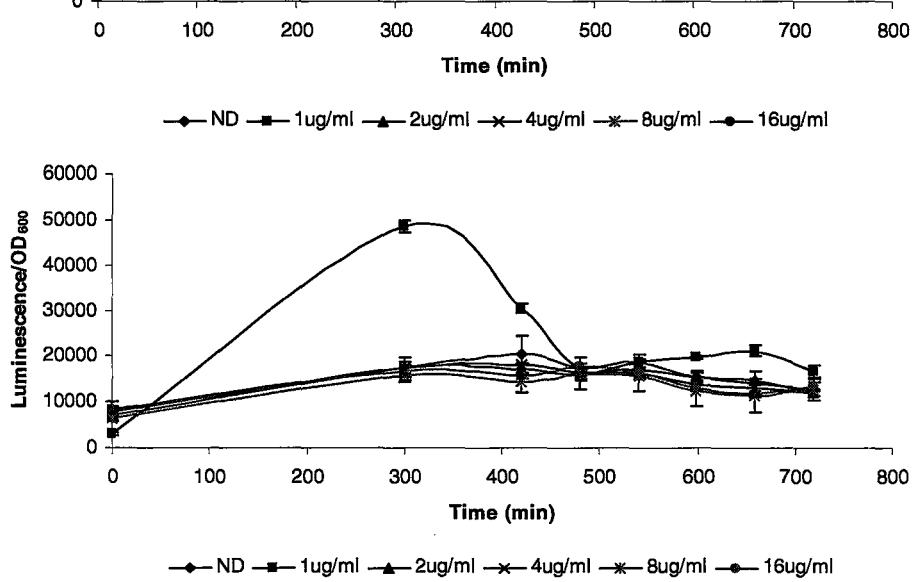
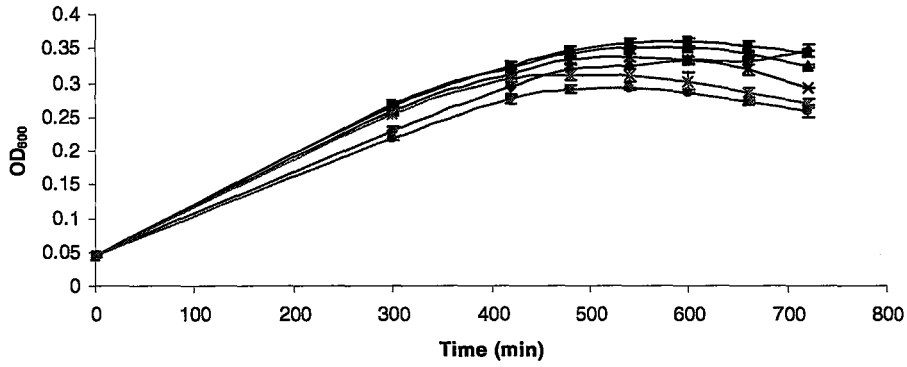
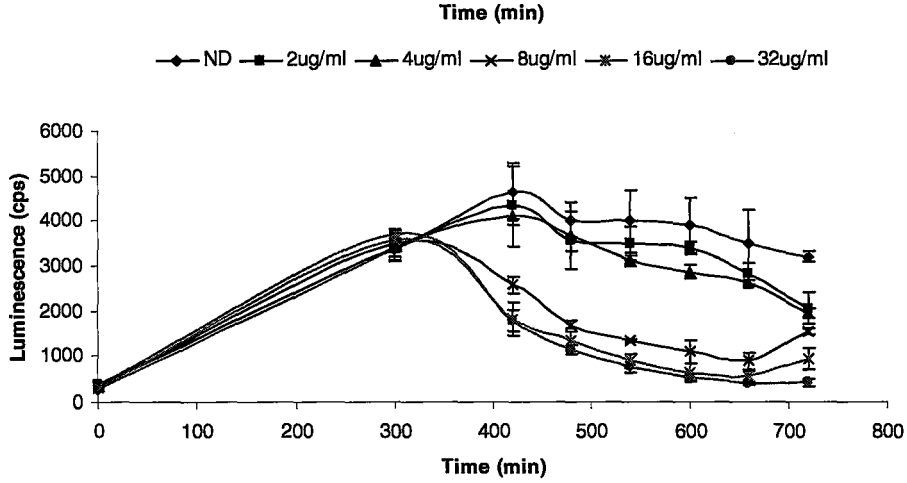


Figure 5.5-7. Effect of nalidixic acid on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Nalidixic acid was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

A



B



C

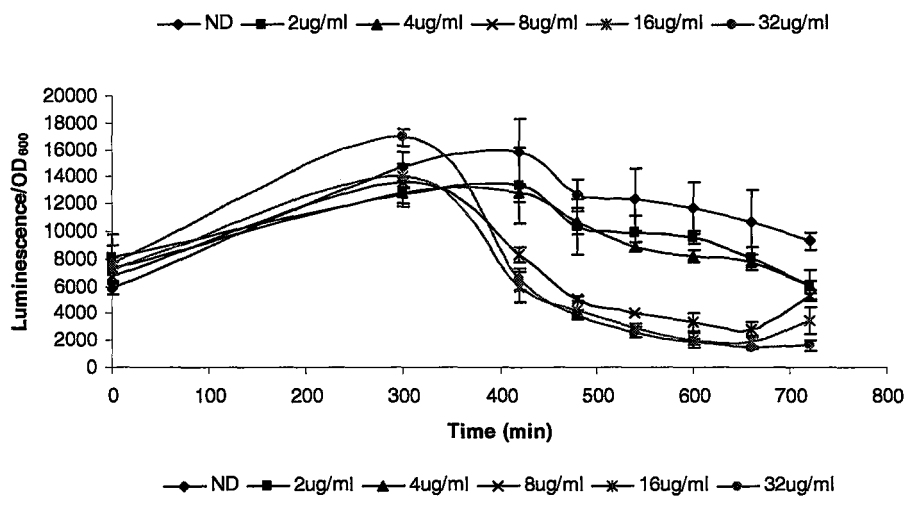


Figure 5.5-8. Effect of neomycin on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Neomycin was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

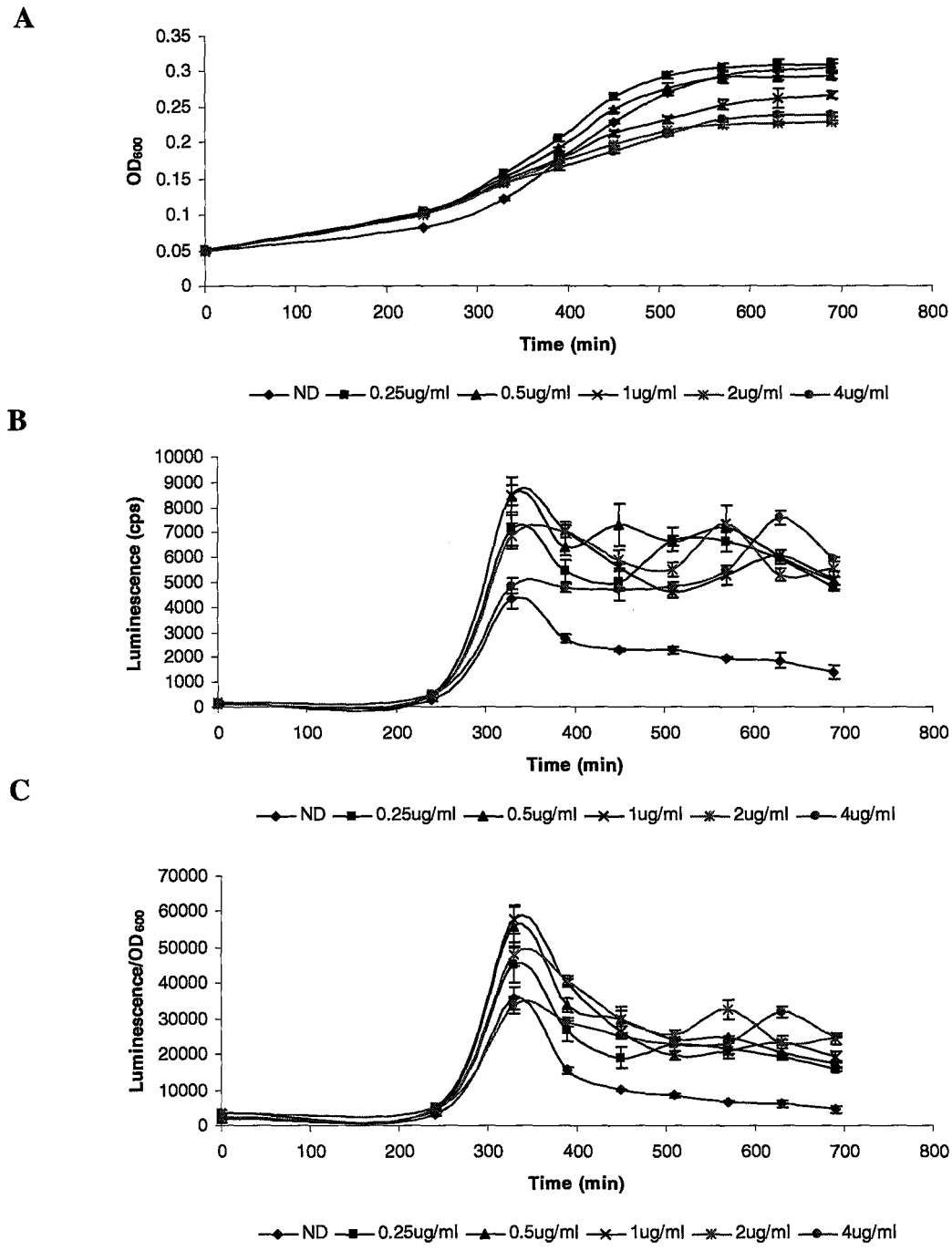


Figure 5.5-9. Effect of novobiocin on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Novobiocin was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

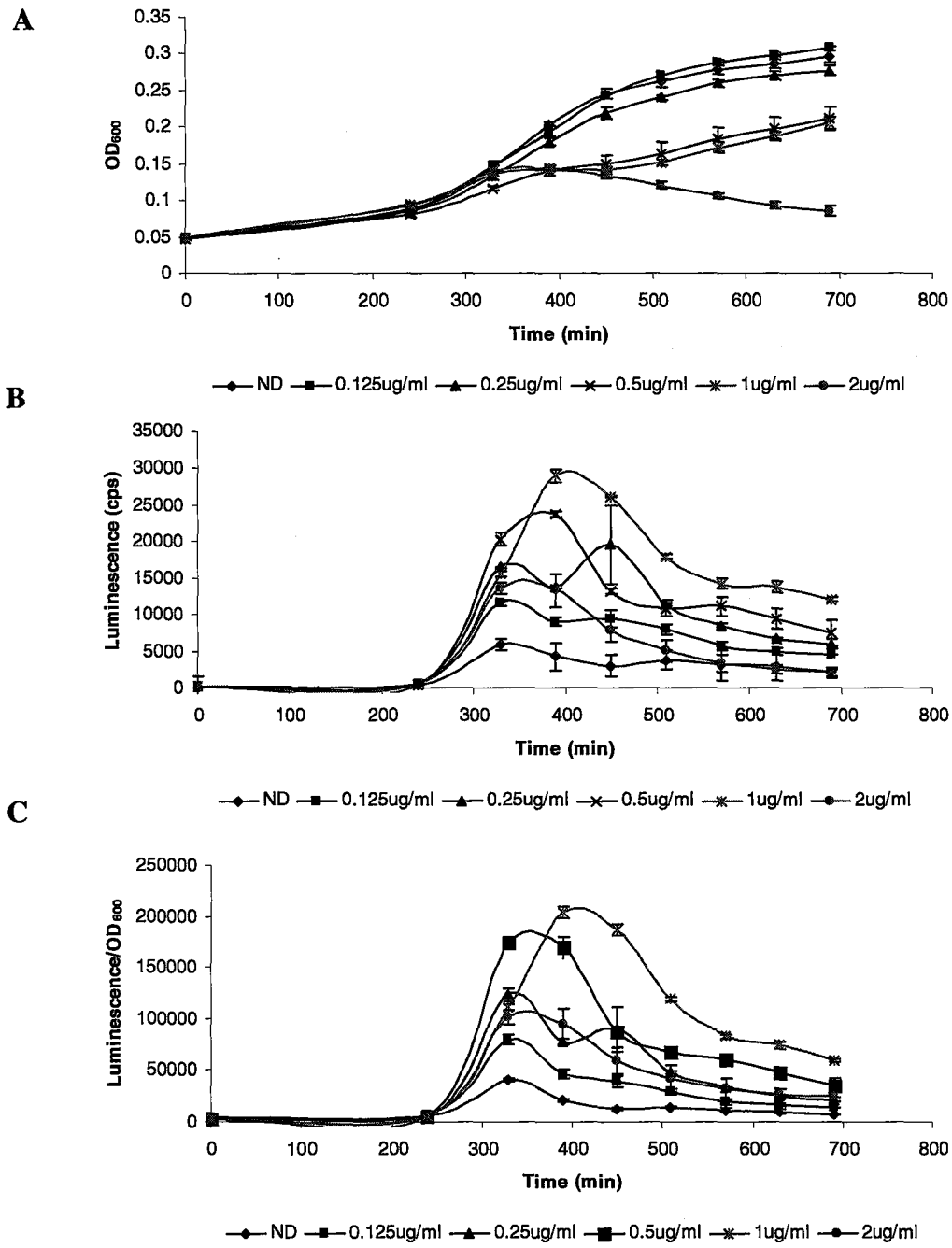


Figure 5.5-10. Effect of ramoplanin on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Ramoplanin was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

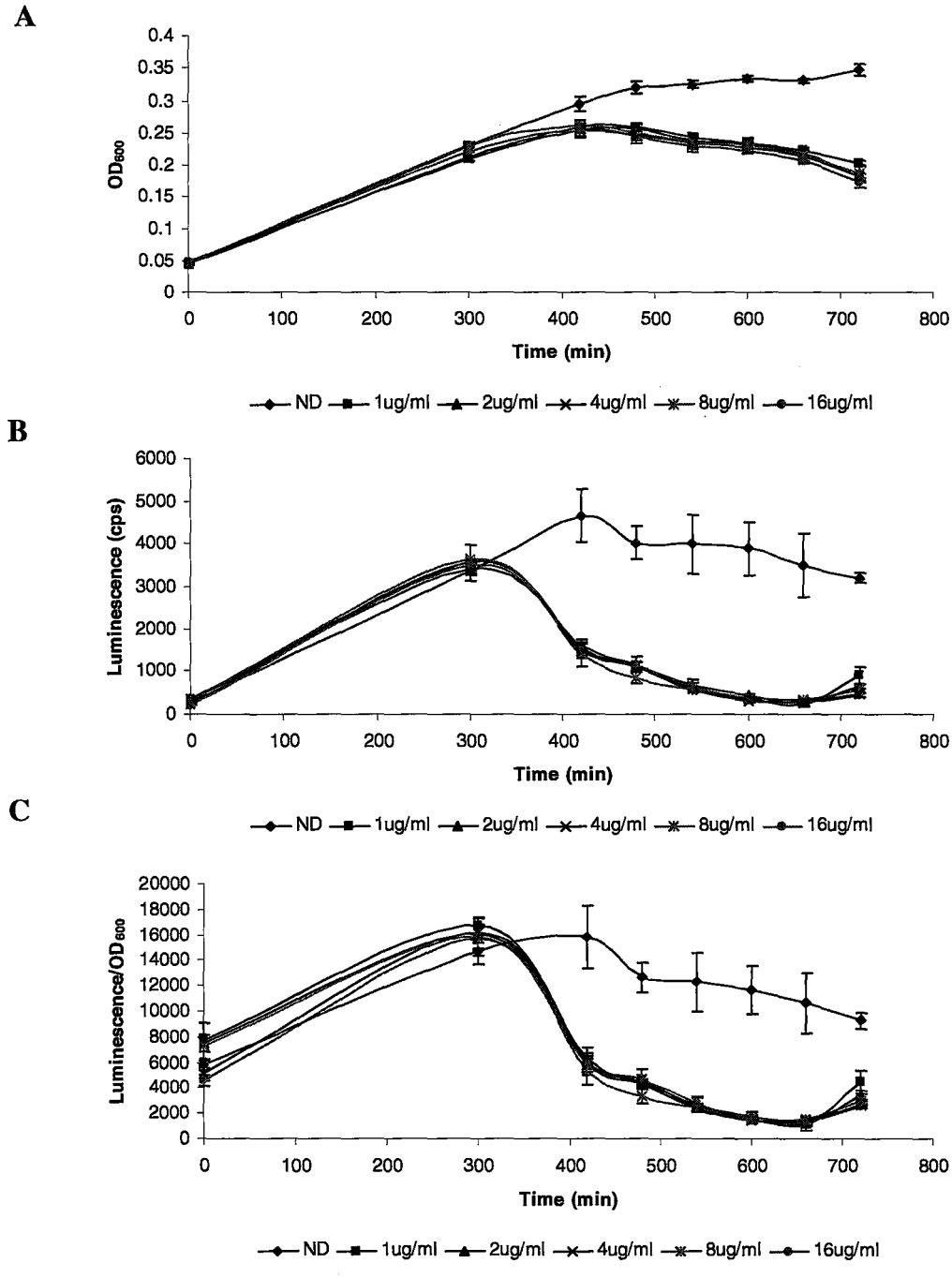


Figure 5.5-11. Effect of tetracycline on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Tetracycline was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

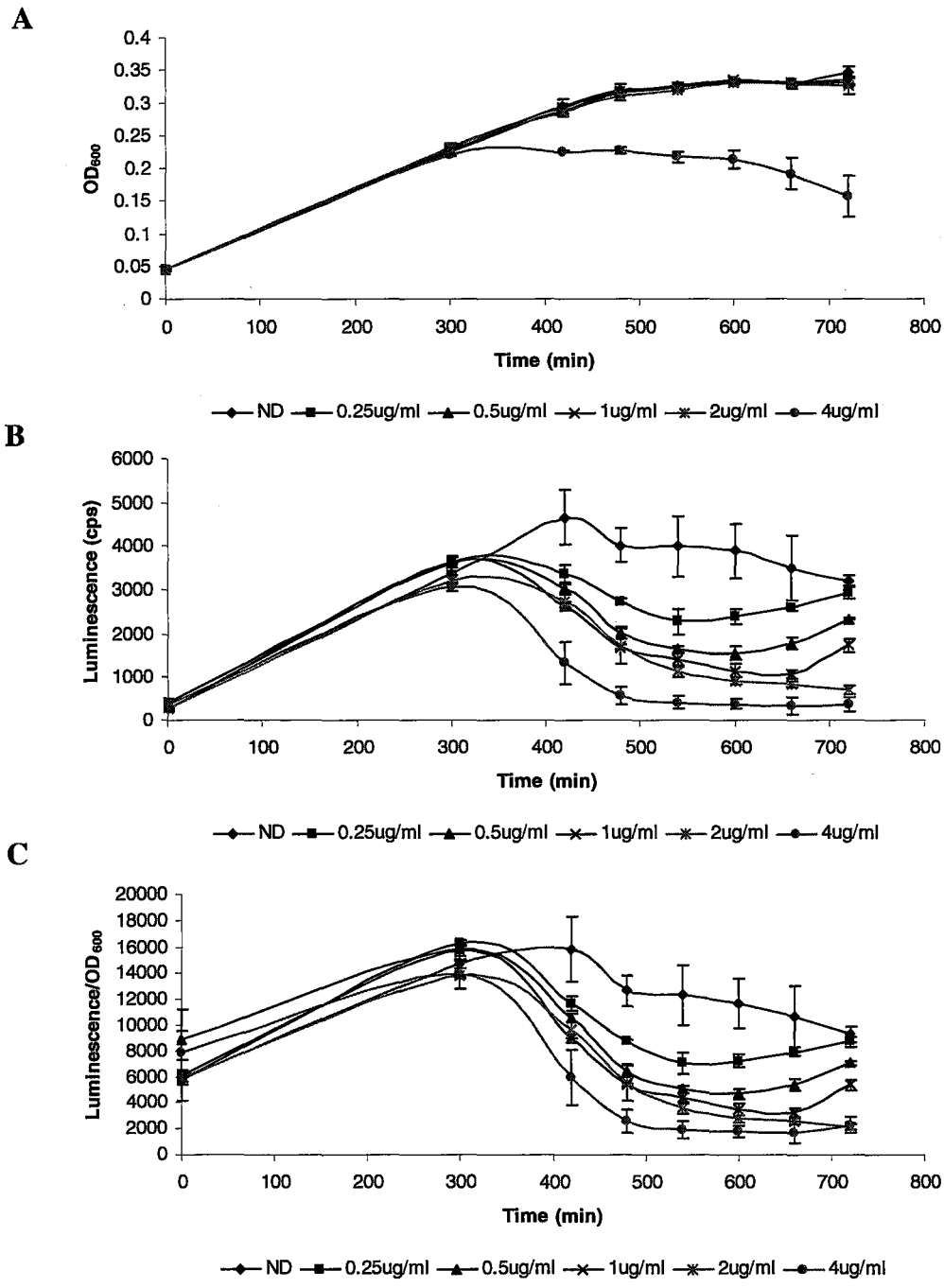


Figure 5.5-12. Effect of triclosan on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Triclosan was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

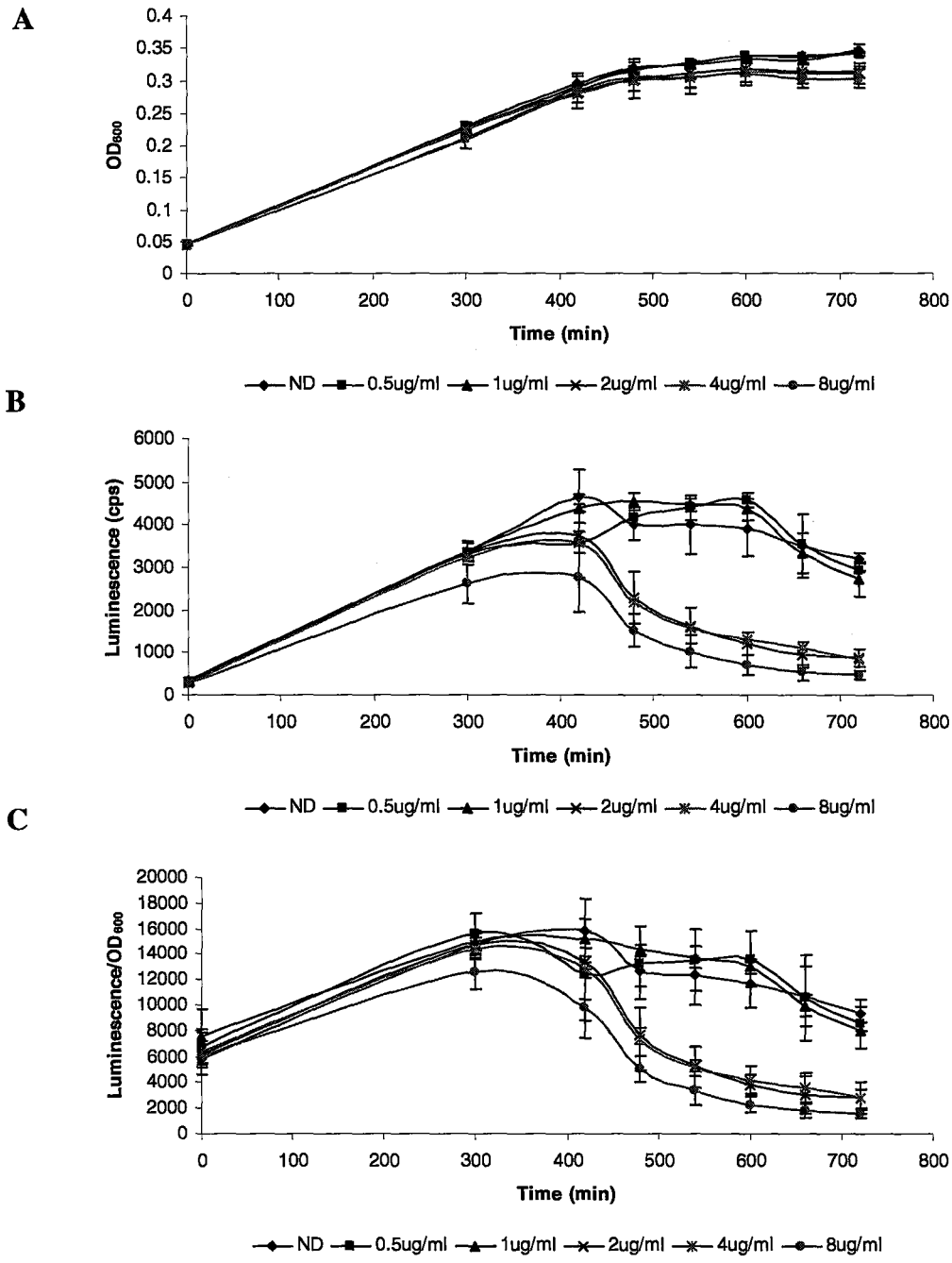


Figure 5.5-13. Effect of trimethoprim on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Trimethoprim was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

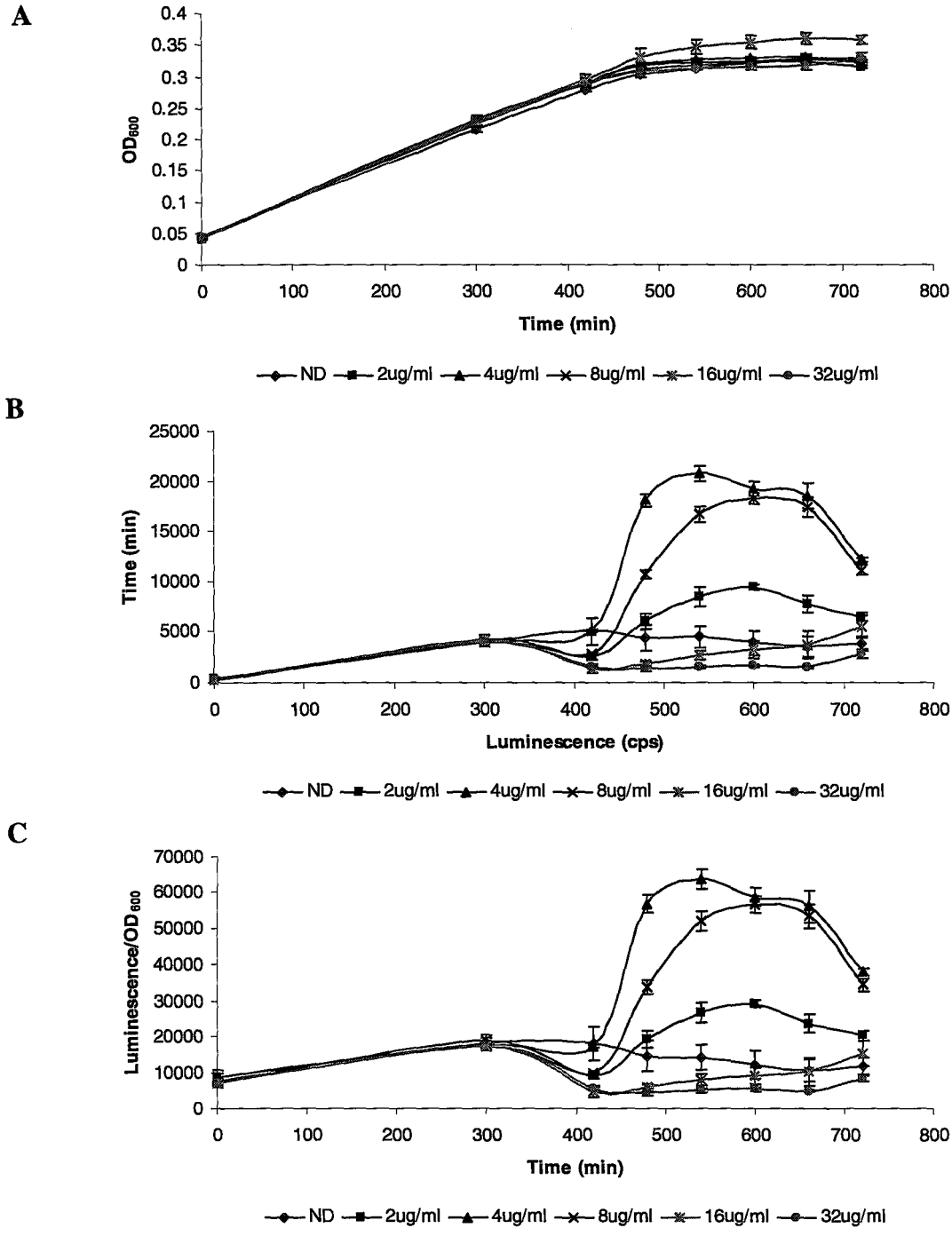


Figure 5.5-14. Effect of tunicamycin on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Tunicamycin was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.

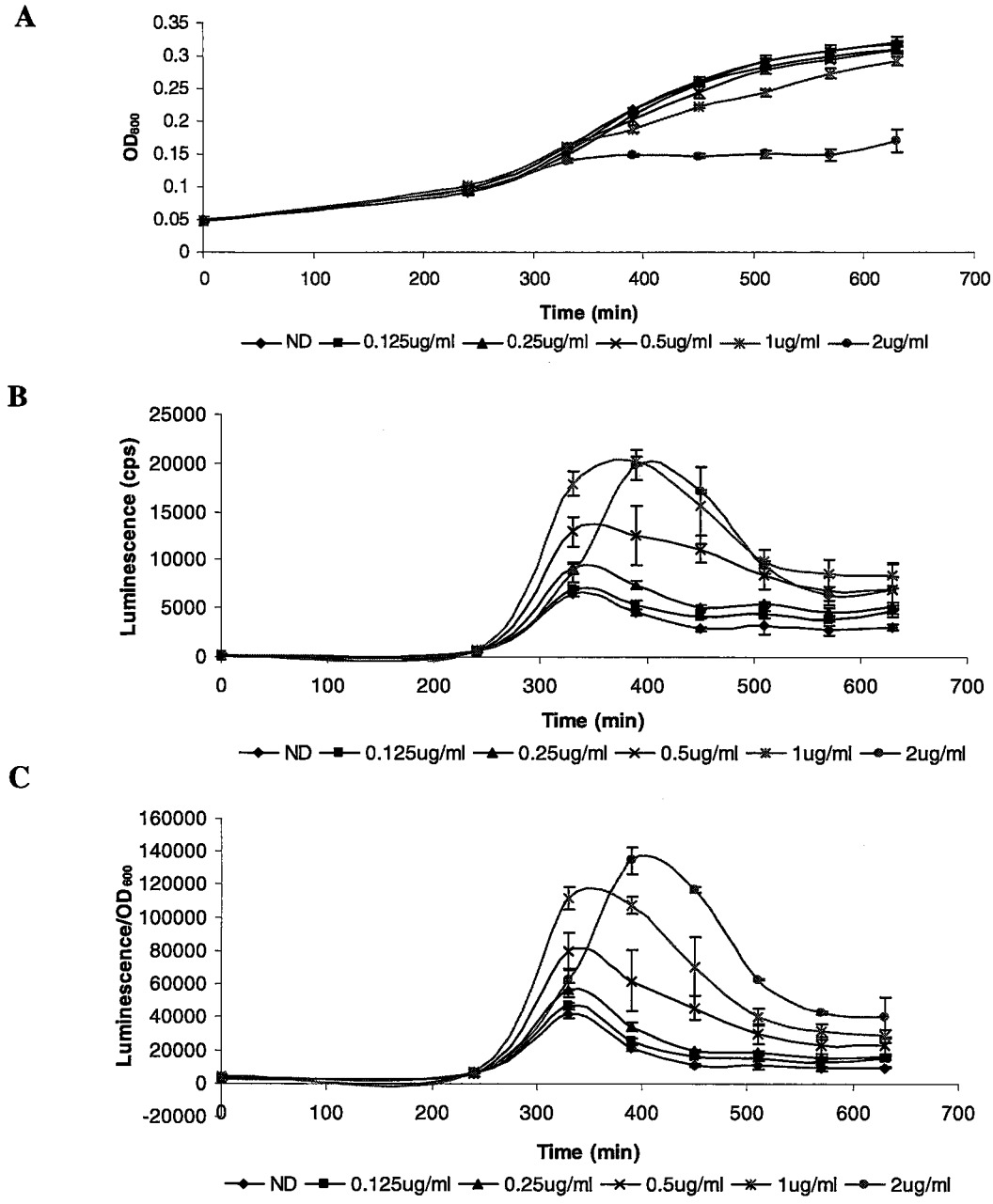


Figure 5.5-15. Effect of vancomycin on the P_{ywaC} reporter strain in the wildtype background (EB6). This strain was grown in LB supplemented with erythromycin. Growth (A) and luminescence (B) were monitored by determining the optical density (OD_{600}) and counts per second (cps) respectively. Normalized luminescence obtained values for the relative number of cells (cps/OD_{600}) (C). Vancomycin was added to the cells after ~ 300 minutes of growth. The experiment was performed in triplicate and the standard deviation was derived from the mean of the data at each time point.