RAMC PRODUCTION BY MUTANTS OF *S. COELICOLOR*

RAMC PRODUCTION BY DEVELOPMENTALLY IMPAIRED MUTANTS OF *STREPTOMYCES COELICOLOR*

BY

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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 (2002) McMaster University

(Biochemistry) Hamilton, Ontario

TITLE: **RamC Production By Developmentally Impaired Mutants of *Streptomyces coelicolor***

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NUMBER OF PAGES: ix, 82

ABSTRACT

The RamC protein is required for the production of spore-forming cells called aerial hyphae in colonies of *Streptomyces coelicolor*. RamC can be detected during the period between 24 and 48 hours following spore germination however there is a dramatic drop in RamC levels thereafter. This could be explained either by the existence of an active means of RamC removal or by the fact that at later time points in the *S. coelicolor* lifecycle non-RamC producing cells vastly outnumber RamC-producing cells. I characterized a large number of *bld* mutants and found that most of them do not produce RamC. In the majority of the *bld* mutants that do produce RamC, we observed the same pattern of accumulation and loss during colony growth as in wildtype colonies. Furthermore, we identified a small number of mutants that produced RamC such that it persisted at detectable levels for a longer duration or only appeared after a substantial delay relative to the wildtype. None of these RamC-producing *bld* mutants was complemented by plasmids containing a cloned *bldM, bldN* or *ramR* gene, mutations in which also cause persistent or delayed RamC production. These results suggest either that there is more than one differentiated cell type within the substrate mycelium or that *S. coelicolor* colonies actively rid themselves of RamC once the protein's biological function has passed.

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to my supervisor, Dr. Justin Nodwell, for the opportunity to work on this project, and for his patience, guidance, enthusiasm and a lot more without which this thesis could never have been finished.

I would also like to thank the members of my defense committee, Dr. Gerry Wright, Dr. Turlough Finan and Dr. Astrid Petrich, for taking time from their busy schedules to give me their advice and comments.

Thanks go out as well to all the members in Nodwell lab for their constant support and assistance. I would especially like to thank Tamara O'Connor and Mike Hudson for their previous research on RamC, and Nancy Sheeler for the *S. coelicolor* chromosomal library construction. Many thanks to Sue MacMillan, Marcus Eccleston, Reem Ali, and Jill Regoeczi for their technical tips and encouragement.

Finally, I must express my deep gratitude to my dad Zijun Zhang, my mom Kaiyu Li, my brother Xiaochuan Zhang and my husband Hongtao Li for their love, their unyielding, unconditional support and encouragement that have made this possible.

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ABBREVIATIONS

A	Alanine
AA	Amino acids
ABC	ATP-binding-cassette
Amp	Ampicillin
ATP	Adenosine triphosphate
CBP	Calmodulin binding peptide
CDA	Calcium-dependent antibiotic
CL	Chromosomal library
Cm	Chloramphenicol
cmc	Critical micelle concentration
DHPC	1,2- Diheptanoyl-sn-Glycero-3-Phosphocholine
DMS	Dimethyl suberimidate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECF	Extracytoplasmic function
EDTA	Ethylenediaminetetracetic acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HRP	Horseradish peroxidase
K	Lysine
kb	Kilobase
kDa	Kilodaltons
LB	Luria broth
NTG	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
PKA	cAMP-dependent protein kinase
PVDF	Polyvinyldene fluoride
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
Spec	Spectinomycin
TM	Transmembrane

1.0 Introduction

Streptomyces are gram-positive filamentous soil-dwelling bacteria that undergo complex physiological and morphological differentiation in their life cycle. They are best known for the production of numerous secondary metabolites, the most notable of which are antibiotics. In addition, at least three distinct cell types, which have specific functions and precise spatial relationships to one another, are developed during the growth of *Streptomyces* (Chater, 1993), and because of this they are also important model systems for studying bacterial differentiation.

1.1 *Streptomyces coelicolor*

Streptomyces coelicolor is the most intensively studied species of *Streptomyces*. The life cycle of *S. coelicolor* commences with germination of a spore and thereafter the substrate hyphae grow by elongation and branching. Cell division during the growth of substrate mycelium occurs infrequently, giving rise to long hyphal cells, some of which contain more than ten copies of the genome. After about two days a second cell type called the aerial hyphae showing a white fuzzy appearance emerges from the surface of colonies. In contrast to substrate hyphal compartments containing many copies of genome, cell-wall extension of aerial hyphae form regularly spaced septa, subdividing them into uninucleoid compartments. The aerial hyphae eventually develop into long chains of gray-pigmented unigenomic spores (Figure 1) (Chater, 1998).

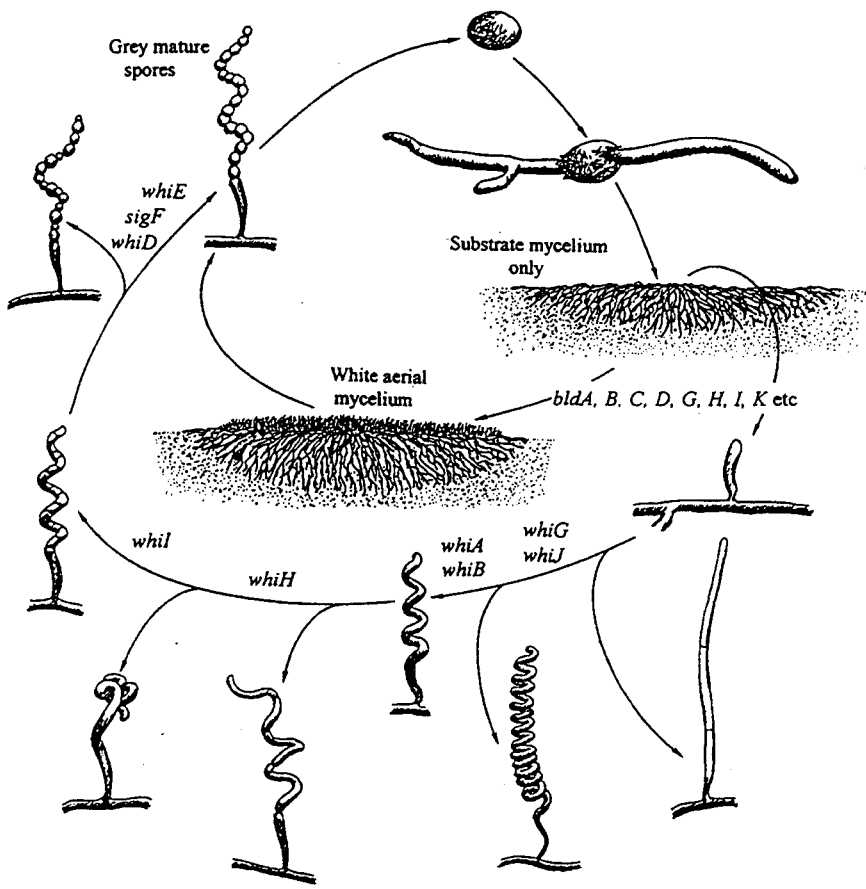


Figure 1. The life cycle of *Streptomyces coelicolor* (Chater, 1998). Phenotypes for developmental mutants are shown as arrows from the normal life cycle immediately preceding the gene name.

The onset of the formation of aerial hyphae is associated with the production of varied secondary metabolites by the substrate hyphae. There are at least four antibiotics produced by *S. coelicolor*: actinohordin which has a blue pigmentation, methylenomycin (encoded by the large plasmid SCP1), undecylprodigiosin which is red and the calcium-dependent antibiotic (CDA).

The genome of *S. coelicolor* has now been entirely sequenced (http:// www.sanger.ac.uk/Projects /S_coelicolor/). It is 8.7Mb long, about twice the length of the *E. coli* genome and contains over 7800 genes (Bentley *et al.*, 2002).

1.2 *whi* genes

More and more genes have been revealed to regulate the formation of aerial hyphae and sporulation in *S. coelicolor*.

The *whi* genes control sporulation within the aerial hyphae. They were originally identified by the *whi* mutants, which erect white aerial mycelium but cannot give rise to the characteristic gray pigment associated to mature spores (Hopwood *et al*, 1970).

The *whi* genes are further subdivided into early and late *whi* genes, depending on the state at which aerial hyphae development is blocked in the mutants. Early *whi* gene mutants prevent the formation of the spore compartments, including the characteristic straight aerial hyphae of *whiG* mutants, abnormally long and coiled aerial hyphae of *whiA* and *whiB* mutants, and loosely coiled and widely spaced aerial hyphae of *whiH* mutant (Figure 1) (Chater, 1975). Late *whi* gene mutants including *whiD, whiE* and *sigF* block

the final stages of sporulation and spore pigmentation. *whiD* (Chater, 1972) mutants form sporulation septa but fail to go on to produce mature spores, *whiE* (Davis & Chater 1990; Yu & Hopwood, 1995) mutant is unable to produce the genes that create mature spores, whereas the spore chains developed in the *sigF* (Potúcková *et al.*, 1995) mutant are thin-walled and poorly pigmented.

Recent research has identified and characterized many *whi* gene products. *whiG* (Chater *et al.*, 1989) encodes a sporulation-specific RNA polymerase sigma factor. *whiG* is epistatic to *whiA, whiB* and *whiH (*Chater, 1975; Flärdh *et al.*, 1999*)*. Additional copies or increased expression of *whiG* cause premature and ectopic sporulation, indicating it is a crucial determinant to initiate the development program that leads to the formation of spore from aerial hyphae (Mendez & Chater, 1987). σ^{WhiG} RNA polymerase holoenzyme transcribes two early *whi* regulatory genes: *whiH* (Ryding *et al.*, 1998) and *whiI* (Ainsa *et al.*, 1999). Both WhiH and WhiI consist of a DNA-binding domain and a domain usually involved in signal sensing. WhiH resembles a family of DNA-binding regulatory proteins responsive to carboxylate-containing intermediates of carbon metabolism (Ryding *et al.*, 1998), whereas WhiI is a homologue to two-component response regulators, however, it lacks two crucial conserved residues from the phosphorylation pocket (Ainsa *et al.*, 1999). WhiA is a protein of unknown function (Ainsa *et al.*, 2000). *whiD* and *whiB* encode small, highly charged proteins and have apparent homologues of unknown function in actinomycetes (Molle & Buttner, 2000). *whiE* gene cluster specifies the polyketide spore pigment (Davis & Chater, 1990), and its transcription is depended on "early" *whi* genes including *whiA, whiB, whiG, whiH, whiI* and *whiJ* (Kelemen &

Buttner, 1998). Apart from WhiG, SigF (Potúcková et al., 1995) is the second RNA polymerase sigma factor required for sporulation in the aerial hyphae of *S. coelicolor*. In contrast to WhiG, which is required for early events of sporulation, and *whiG* mRNA was present at all time points, including those taken prior to aerial mycelium formation, SigF is proposed to control the late stages of spore development. *sigF* transcripts were detected transiently when sporulation septa were observed in the aerial hyphae. Transcription of *sigF* depended upon all six of the early *whi* genes (*whiA, B, G, H, I* and *J*), including *whiG*. The dependence of *sigF* transcription upon *whiG* is very likely to be indirect (Kelemen *et al.*, 1996).

1.3 *bld* genes

Mutants of *S. coelicolor* that fail to form aerial hyphae are traditionally referred to as *bld* (bald) mutants. Usually they show a smooth, "bald" appearance (Merrick 1976). A shared trait of some *bld* mutants *(bldA, bldC, bldD, bldG, bldM, bldK)* is that their defects in aerial hyphae formation are carbon source dependent. They are bald on glucose minimal media but form aerial hyphae and even sporulate on the media with carbon sources such as mannitol or galactose. This carbon source conditional feature of *bld* genes implies an alternative pathway in the morphological differentiation.

Many *bld* gene mutations, including *bldA, bldB, bldD, bldG, bldH* and *bldJ*, also cause a defect in antibiotic production. This indicates that there are regulatory links between the formation of aerial hyphae and secondary metabolites. Indeed, these two events are initiated at the same time during the life cycle of wildtype *S. coelicolor*.

It is believed that the formation of aerial hyphae in *S. coelicolor* involves intercellular signaling. Growing certain pairs of *bld* mutants in close proximity to each other can restore the formation of aerial hyphae, which is believed to be mediated by intercellular signals. The extracellular complementation is unidirectional; one mutant acting as a donor while the other as an acceptor. Each mutant can restore aerial hyphae formation to all members of the groups listed to its left but to none of the groups listed to its right (see below). An extracellular complementation hierarchy was suggested up to 5 intercellular signals (Willey *et al.*, 1993, Nodwell *et al.*, 1996, 1999, Nodwell & Losick, 1998, Molle & Buttner, 2000).

bldJ < *bldK* / *bldL* < *bldA* / *bldH* < *bldG* < *bldC* < *bldD* / *bldM*

All *bld* mutants were correlated to the failure to produce the morphogenetic protein SapB, a small protein that appears to be directly involved in erecting aerial hyphae (Chater 1993). SapB has been shown to act as a surfactant and reduce the water surface tension and facilitate the emergence of aerial hyphae at the colony-air interface (Willey *et al.* 1991, Tillotson *et al.* 1998). The addition of purified SapB can induce transient aerial mycelium formation of *bld* mutants (Willey *et al.*, 1993). Thus, the SapB production is assumed to occur either directly or indirectly under the control of *bldD* and suggested to be the final step of the signaling cascade (Nodwell *et al.*, 1996).

So far, only one such extracellular signaling molecule has been proven to exist in *S. coelicolor* and it is imported by a *bldK* encoded oligopeptide permease. *bldK* is a complex locus that encodes protein homologous to ATP-binding-cassette (ABC)

membrane-spanning transporter homologous to the product of the *spo0K* gene clusters of *B. subtilis*. Like Spo0K, BldK permease has been revealed to be capable of importing signaling molecules (Nodwell *et al*, 1996). One of the candidate molecules has been purified but not yet characterized in molecular detail (Nodwell & Losick, 1998).

However, the regulation of morphogenesis in *S. coelicolor* is not likely to be a simple linear cascade, as many *bld* genes do not fit into this extracellular complementation cascade.

Several other genes are also believed to be associated with extracellular signals. The *ramA* and *ramB* genes also encode subunits of a ABC transporter. They share significant carboxy terminal homology with some export proteins, and in contrast to BldK, they probably export a signaling molecule (Ma & Kendall, 1994). CprA appeared to act as an activator for secondary metabolism and morphogenesis in *S. coelicolor*, it showed about 35% identity to A-factor receptor protein AprA of *Stretomyces griseus* (Onaka *et al.*, 1998). In *S. griseus*, A-factor (2-iso-capryloyl–3R–hydroxymethyl-γ-butyrolactone) is identified as a microbial hormone, which controls a set of biological activities including aerial mycelium formation at a very low concentration (10^{-9} M) (Khokhlov *et al.*, 1967; Horinouchi & Beppu, 1994). AprA is therefore presumed to bind A-factor and activate morphogenetic genes (Miyake *et al.*, 1989; Onaka *et al.*, 1995). A-factor binds ArpA and dislocates it from DNA releasing repression of *adpA* gene. AdpA protein then goes on to activate antibiotic genes. It is not known whether a similar cascade of events occurs in *S. coelicolor* under the control of CprA.

S. coelicolor has abundant two-component regulatory systems. Two-component regulatory system consists of a cytoplasmic membrane associated sensor and a cytoplasmic response regulator. Usually, the sensors belong to a family of histidine protein kinases and they respond to environmental signals by autophosphorylation at conserved histidine residues. This mechanism requires formation of a homodimer with one kinase monomer catalyzing the transfer of γ-phosphate to a histidine residue in a second monomer (Yang & Inouye, 1992; Ninfa *et al.*, 1993; Wolfe & Stewart, 1993; Swanson *et al.,* 1993; Pan *et al.,* 1993). The response regulator typically contains an amino terminal receiver domain and one or more carboxyl terminal output domains that are joined by flexible linkers. The phosphoryl group of phosphohistidine in sensor kinase is then transferred to a conserved aspartate in the receiver domain of the response regulator. And the phosphorylation state of the receiver domain controls the activity of the output domain to trigger an adaptive response. The output domains usually have DNA binding or other regulatory transcriptional function over target genes. The entirely sequenced genome of *S. coelicolor* revealed 85 sensor kinases and 79 response regulators, including 53 sensor-regulator pairs (Bentley *et al.*, 2002). Among *bld* genes, *bldM* (Molle & Buttner, 2000) and *ramR* (Ma & Kendall, 1994; Keijser *et al.*, 2002; O'Connor *et al.*, 2002) have been identified to be response regulators of the two-component regulatory system. Interestingly, the phosphorylation on aspartate of *bldM* may not be necessary for its function (Molle *et al.,* 2000), suggesting it is an unusual response regulator. Regardless of whether this is the case, cognate sensor kinases for both BldM and RamR are still not identified.

Other identified *bld* genes include *bldN, bldG, bldD* and *bldB*. The gene *bldB* encodes a 98 amino acid protein with unknown function (Pope *et al.*, 1998). Recent research suggests that BldB interacts with itself, probably to form a dimer, in which amino acid residues 20-78 play an important role (Eccleston *et al.*, 2002). BldD (Elliot et al., 2001) is revealed to bind and repress promoters of *bldN* and *whiG*. As mentioned in *whi* genes, *whiG* is a early *whi* gene encoding a sigma factor and its mRNA was present as early as prior to aerial mycelium formation (Kelemen *et al.,* 1996). This is the first time that a regulatory link is suggested between *bld* and *whi* genes, however, the developmental importance of this relationship needs to be clarified. *bldN* was indicated to encode a member of the extracytoplasmic function (ECF) subfamily of RNA polymerase sigma factors, however, with an amino terminal extension of 86 residues that is absent in other sigma factors (Bibb *et al.*, 2000). Furthermore, sigma factor BldN transcribes *bldMp1*, the stronger promoter of two *bldM* promoters. *bldG* encodes an anti-anti-sigma factor that regulates the activity of sigma factors (Bignell *et al.*, 2000).

1.4 RamC

1.4.1 Another gene required for the formation of aerial hyphae is *ramC*

ramC is another gene required for the formation of aerial hyphae in *S. coelicolor* (O'Connor *et al.*, 2002; Hudson *et al*, 2002). It is a member of *ram* gene cluster (Figure 2), which is composed of *ramC, ramA, ramB* and *ramR* (mentioned above), and *ramS*, which encodes a small protein with unknown function. RamR protein interacts directly with DNA in the *ramC* promoter region suggesting that it regulates the *ramC* expression (O'Connor *et al.*, 2002).

Figure 2. Organization of the *ram* gene cluster. *ramCSAB* are transcribed in the same direction, while *ramR* is transcribed in the opposite direction.

While two-component regulatory systems are ubiquitous in prokaryotes; the serine/threonine and tyrosine kinase cascades are more commonly associated with eukaryotes. Similar to histidine sensor kinase in two-component systems, the serine/threonine and tyrosine kinases respond biochemically to external stimuli by autophosphorylation, however, in this case on serine, threonine and tyrosine residues. Autophosphorylation may result in a conformational change that allows better access of exogenous substrates to the active site. Therefore, the active (phosphorylated) serine/threonine and tyrosine kinases bind to target protein and ATP, transfer the γ-phosphate of ATP to the serine, threonine or tyrosine residue of target protein. Again, the phosphorylation on target proteins alters their activity and brings about a series of intracellular responses. Recent research revealed several novel serine/threonine kinases in prokaryotes (Bakal & Davies, 2000). Among them, AfsK is a well studied, possibly membrane associated protein serine/threonine kinase in *S. coelicolor*. Amino acid

sequence of AfsK shows significant similarity to the catalytic domains of eukaryotic serine/threonine kinases. Upon autophosphorylation on serine and threonine, AfsK thereafter phosphorylates adjacent regulatory protein AfsR at both serine and threonine residues (Matsumoto *et al.*, 1994). AfsK was found to control the secondary metabolism in the *S. coelicolor* A3(2) (Matsumoto *et al.*, 1994), and it restored the aerial mycelium formation in the A-factor-deficient strain *S. griseus* HH1 (Ueda *et al.*, 1996). In addition, an AfsK mutant that loses kinase activity was incapable of inducing aerial mycelium in *S. griseus* (Ueda *et al.*, 1996), indicating that the kinase activity of AfsK is important in the regulation of morphological differentiation.

RamC is also a membrane associated predicted serine/threonine kinase identified in *S. coelicolor* (Hudson *et al.*, 2002; O'Connor *et al.*, 2002). RamC is crucial for aerial mycelium formation in *S. coelicolor* (Figure 3A) but dispensable for the antibiotic production (O'Connor *et al.*, 2002). Amino acid sequence analysis indicated that RamC protein consists of several parts: the amino-terminal half of the protein, potential transmembrane domains (TM), repeat domain and a possible "sensory domain" of unknown function (Figure 3C) (Mike Hudson, unpublished data). The amino terminal half of RamC shows some homology to some other serine/threonine protein kinases including cAMP-dependent protein kinase (PKA), the *S. coelicolor* kinase AfsK, the *S. toyocaensis* kinase StoPK1, and the *S. griseus* RamC homologue AmfT. These serine/threonine kinases are resembled by several highly conserved amino acid sequences, which were originally identified by Hanks *et al.* (1988) and are therefore named Hanks domains. Several parts of amino acid sequences of the RamC amino

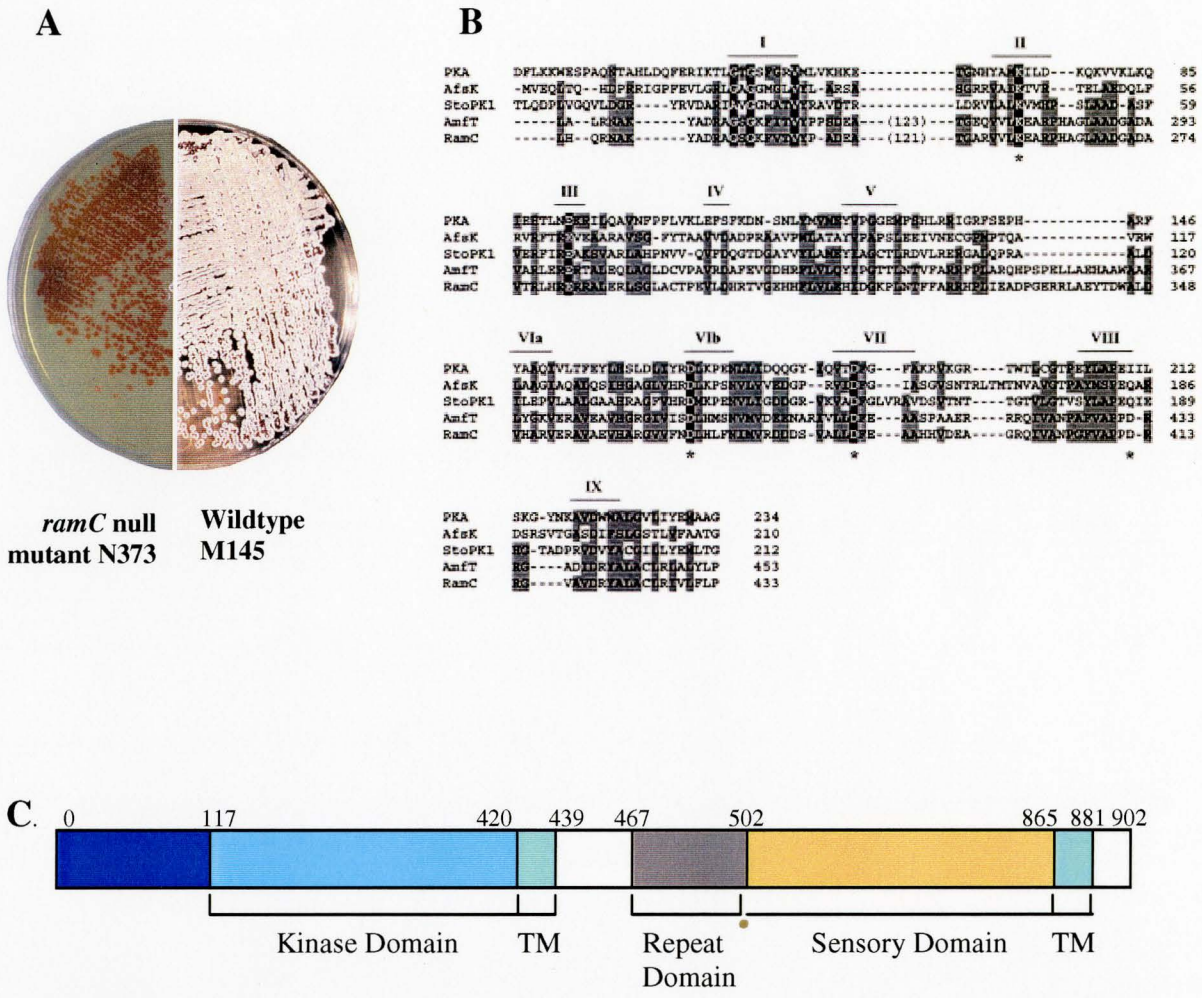


Figure 3. RamC is essential for the formation of aerial mycelium in *S. coelicolor* and identified as a potential membrane associated serine/threonine protein kinase. (A) *ramC* null mutant N373 is unable to form the white aerial hyphae that seen in wildtype strain M145 (Tamara O'Connor, unpublished data). (B) Sequence alignment of RamC with a cAMP-dependent protein kinase (PKA), the *S. coelicolor* kinase AfsK, the *Streptomyces toyocaensis* kinase StoPK1, and the *S. griseus* RamC homologue AmfT. Residues shaded in grey denote similarity to RamC; those shaded in black are predicted, on the basis of well-known model kinases, to be involved in catalysis of phosphorylation. Conserved kinase-like sequence motifs ("Hanks" motifs) are indicated by roman numerals according to convention. Asterisks indicate the point mutations introduced into RamC (Hudson *et al.*, 2002). (C) The diagrammatic representation of the putative domains of RamC protein. (Mike Hudson, unpublished data) (TM – transmembrane domain)

terminus are homologous to Hanks domain II, III, VIb and VII (Figure 3B). In general, the domain II and III of Hanks type kinases bind to the nucleotide triphosphate that serves as phosphoryl donor, and the VIb and VII domains bind the substrate proteins and transfer the γ-phosphate to the substrate (Zheng *et al.*, 1993).

Furthermore, some amino acid residues conserved in Hanks kinases that are believed to play important roles in catalysis are also found in RamC. They either function to correctly position ATP for phosphate transfer (such as lysine72, aspartate 184 in PKA) or deprotonate the target hydroxyl on the target protein (such as aspartate166 in PKA), facilitating its nucleophilic attack on the γ-phosphate (Zheng *et al.*, 1993). The amino-half of RamC also contains these conserved residues, they are lysine(K)259, aspartate(D)387 and aspartate(D)369. The mutations of these residues with an alanine, including K259A, D369A and D387A, were prepared on pTO8, a *ramC* complementation plasmid that contains a cloned *ramC* gene. When introduced into a *ramC* null mutant, plasmids pTO8-K259A, pTO8-D369A and pTO8-D387A lost their complementation ability, indicating that these three residues in the amino terminal half of RamC were indeed essential for its function (Hudson *et al.*, 2002). Therefore, the amino terminal half of *ramC* is believed to encode a membrane-associated receptor kinase. Autophosphorylation on serine, threonine and tyrosine residues are common in Hanks kinases; it alters their conformation and thereby facilitates the subsequent phosphorylation on target proteins. However, autophosphorylation by RamC has not yet been convincingly demonstrated.

1.4.2 The expression timing and pattern of RamC in *S. coelicolor*

More interestingly, RamC is expressed predominantly in the substrate hyphae and dormant in spores (O'Connor *et al.*, 2002). This expression pattern of RamC is so far unique among the development genes in *S. coelicolor*. Western analysis shows that RamC accumulates in the first 24 hours of colony development, peaks at 36 hours, drops at 48 hours and that the protein disappears completely between 48 hours and 72 hours. RamC is expressed under the direct activation by response regulator RamR. However, *ramR* null mutant does produce RamC at a much later time point than wildtype, suggesting an alternative pathway that activates *ramC* expression. To date, genes required for *ramR*-independent activation of *ramC* have not been identified. In addition, RamC expression is dependent on *bldD* and *cprA*, but not *bldM* and *bldN* (O'Connor *et al.*, 2002).

Since *ram*C was active primarily in the substrate hyphae, it is possible that *whi* genes, which regulate the sporulation in *S. coelicolor*, might negatively regulate its expression in aerial hypae. If it is the case, the null mutation in these genes might cause overproduction of RamC, and the RamC protein might persist for a longer time than it appeared to do in wildtype colonies. The accumulation pattern of RamC in *whiA, whiB, whiG, whiH* and *sigF* null mutants was tested by Western analysis (unpublished data by Tamara O'Connor). The results revealed that the accumulation of RamC in these strains were very similar to wildtype *S. coelicolor*, RamC is detectable at 24 hours post germination, increases between 24 and 36 hours, and then decreases thereafter and drops

to undetectable level between 48 and 72 hours. It suggests that the drop in RamC level is independent of sporulation events that follow the erection of the aerial mycelium.

1.5 Objective

The cause of the disappearance of RamC during the aerial mycelium formation of *S. coelicolor* is not clear. One possibility is that the ratio of RamC-producing substrate hyphae to non-RamC producing aerial hyphae drops during the aerial hyphae development, so that the overall RamC protein in the whole cell extracts drops to an undetectable level. An alternative hypothesis could be that RamC is proteolytically destroyed once its biological function has passed in that the cells that produce it die and are consumed by the rest of the cells. There is little precedent for either of these phenomena in the molecular biology of developing prokaryotes and this is the question that I have sought to address in this work.

RamC production and accumulation was investigated in a large collection of mutants that are defective in aerial mycelium formation, including 5 well characterized *bld* mutants *bldB, bldC, bldG, bldK, bldI* and 138 unidentified *bld* mutants that were generated by NTG (*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine) mutagenesis (Nodwell *et al.*, 1999).

Based on the RamC production pattern, 143 *bld* mutants were divided into 3 groups. Most *bld* mutants eliminated or dramatically reduced RamC production, which includes *bldB, bldC, bldG, bldK, bldI* and another 98 unidentified *bld* mutants. However, 33 *bld* mutants caused little or no alteration in RamC production, implying that the

apparent loss of RamC in wildtype *S. coelicolor* was independent of aerial mycelium formation, and probably not simply due to the cells in non-RamC producing aerial hyphae outnumbering those in RamC-producing substrate hyphae. Or alternatively, there are two differentiated cell type exists within the substrate hyphae rather than one. As colony growth proceeds, the RamC-producing cells outnumber those non-RamC producing cells. The smallest group consists of 7 unidentified *bld* mutants; they produced RamC in a manner that primarily altered time of appearance, including mutants that like *ramR* produce RamC after a lengthy delay or others that like *bldM* and *bldN* produced RamC that appeared to persist for a longer period of time than in the wildtype. None of these 7 mutants was complemented *in trans* by a *bldM, bldN* and *ramR* gene. They are potential novel *bld* mutants and it is possible that some *bld* mutants in this group contain the mutation in the genes which encode proteins directly or indirectly involved in the RamC removal. In the future, the chromosomal library of *S. coelicolor* could be used for complementing these mutants.

Plasmids containing a gene *bldC* and *ramC* were used to complement 18 unidentified *bld* mutants. Only two *bld* mutants were induced to form the aerial hyphae by introduction of a *bldC* and *ramC* gene. Furthermore, none of the 33 unidentified *bld* mutants that caused little or no alteration in RamC production were complemented *in trans* by a *bldM* or *bldN* gene. These results suggested that many novel *bld* gene mutations existed in these 138 *bld* mutants.

2.0 Materials and Methods

2.1 Materials

Antibiotics and lysozyme were purchased from Sigma. Polyvinylidene fluoride (PVDF) transfer membrane was purchased from Pall Corporation. Western blot chemiluminescence reagent was purchased from PerkinElmer. Horseradish peroxidase linked anti-rabbit IgG was purchased from Amersham Pharmacia.

2.2 Strains and growth conditions

All plasmids were replicated in non-methylating *E. coli* strain ET12567/pUZ8002 (MacNeil *et al.*, 1992) for conjugation into *Streptomyces coelicolor* strains. *Streptomyces coelicolor* strains were grown at 30°C on R2YE for analysis of phenotype and RamC production, MS agar and MS agar supplemented with 10mM $MgCl_2$ for conjugation (Kieser *et al.*, 2000). Following conjugation with *E. coli* (Kieser *et al.*, 2000), exconjugants were selected by overlaying MS Agar + 10mM $MgCl_2$ plates with nalidixic acid (25μg/ml) and apramycin (50μg/ml) or spectinomycin (250μg/ml). Single colonies of exconjugants were restreaked on R2YE supplemented with nalidixic acid (25μg/ml) and aparamycin (50μg/ml) or spectinomycin (250μg/ml), and restreaked on R2YE plates with no antibiotics for phenotypic analysis. *E. coli* strains were grown on solid or in liquid Luria Broth medium at 37°C for plasmid cultivation. Chloramphenicol, kanamycin, apramycin and spectinomycin were used at a final concentration of 25, 25, 50 and 100 μg/ml respectively.

2.3 Western analysis of RamC expression

RamC expression was assessed by Western blot analysis. Hyphal fragments of all mutant and wildtype strains were plated on porous cellophane on top of solid R2YE medium. Mycelium (100mg) was then harvested at 24, 36, 48 and 72 hours intervals thereafter, suspended in 1ml P-buffer (Kieser *et al.*, 2000) containing 2mg/ml lysozyme and incubated at 30°C for 2 hours. A 1 min centrifugation at 18,000 ×g was applied after the treatment with lysozyme. The supernatants of the samples were decanted and the cell pellets were resuspended in 400µl 1.5× SDS-PAGE loading buffer (O'Connor *et al.*, 2002) and boiled for 15min. The mycelium debris were separated from the whole cell lysate (supernatant) by another 1min centrifugation at 18,000 ×g and discarded. Whole cell lysate 30µl that suspended in SDS loading buffer, corresponding to 7.5 mg mycelium, was loaded on 8% SDS-polyacrylamide gel and electrophoresed at 170 volts for 1.5 hours. Proteins were transferred to PVDF transfer membrane at 90 volts for 1 hour and probed with affinity purified anti-RamC antibody and HRP-linked anti-rabbit secondary antibody sequentially as described previously (O'Connor *et al.*, 2002). To ensure that equivalent amounts of protein had been loaded in each case, the protein immobilized on PVDF membranes was stained with Coomassie Blue after chemiluminescence development.

2.4 Complementation analysis

pSET-based complementation plasmids were introduced into *S. coelicolor* strains by conjugation from *E. coli* strain ET12567/pUZ8002 using standard procedures (Kieser *et al.* 2000). The mycelial fragments of all *S. coelicolor* wildtype and mutant strains were

harvested from 3 days grown MS agar plates for conjugation. Single exconjugants were restreaked on R2YE plates containing 25μg/ml nalidixic acid and appropriate complementation plasmid selection, and restreaked again on R2YE plate with no antibiotics for phenotypic analysis.

Table 1 Strains and plasmids

Stains/Plasmid	Description	Background	Reference or source
Streptomyces coelicolor			
1147	Prototroph		Kieser *et al.* (2000)
M145	Prototroph SCP1$^-$, SCP2$^-$		Kieser *et al.* (2000)
J1501	*hisA1 ura A1 strA1 pgl-1* SCP1$^-$ SCP2$^-$		Kieser *et al.* (2000)
J2151	*bldM::Hyg^r* Δ*glkA119* SCP1$^-$ SCP2$^-$		Molle & Buttner. (2000)
J2177	*bldN::Hyg^r* Δ*glkA119* SCP1$^-$ SCP2$^-$		Bibb *et al.* (2000)
N373	*ramC::aac(3)IV* SCP1$^-$ SCP2$^-$		O'connor *et al.* (2002)
N17	*bldK::aadA* SCP1$^-$ SCP2$^-$		Nodwell *et al.* (1996)
N13	*mthB2 cysD18 bldC18 NF SCP2**		Willey *et al.*, (1993)
N25	*bldG103 hisA1 ura A1 strA1 pgl-1* SCP1$^-$ SCP2$^-$		Champness *et al.* (1988)
N985	*bldB::aphI* SCP1$^-$ SCP2$^-$		Eccleston *et al.* (2002)
N376	*ramR::aac(3)IV* SCP1$^-$ SCP2$^-$		O'connor *et al.* (2002)
Escherichia coli			
ET12567/pUZ8002	*dam13::Tn9 dcm6 hsdM hsdR recF143 galK2 galT22 zjj201:: Tn10 ara14 lacY1 syl5 leuB6 thi1 tonA31 rpsL136 hisG4 tsx78 mtl-1 glnV44 F' pUZ8002*		MacNeil *et al.* (1992)
DY330	W3110 Δ*lacU169 nadA::Tn10 gal490 λc1857*Δ(cro-bioA)		Yu *et al.*, (2000)
DHP1	F$^-$ *cya glnv44*(AS) *recA endA1 gyrA96* Nalr *thi-1 hsdR17 spoT1 rfbD1*		Karimova *et al.*, (1998)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 glnV44 hsdR17 supE44 relA1 lac* [F' *proAB lac1*q Z_*M15* Tn*10*(Tetr)]		Stratagene
Er^2-1	F' *lacI leuB6 thi-1 fhuA31 lacY1 tsx-78 galK2 galT22 supE44hisG4 rpsL136*(Str) *xyl-5 mtl-1 dam-13*::Tn*9*(Cam^r) *dcm-6mcrB1 mcrA hsdR2(r$_k^-$ m$_k^+$)*		J. McCormick
Plasmids			
pIJ6602	*bldM*	pSET152	Molle&Buttner. (2000)
pIJ6715	*bldN*	pSET152	Bibb *et al.* (2000)
pIJ6836	*bldC*	pSET152	Buttner lab collection
pSETΩpcs	*ramC ramS aadA*	pSETΩ	O'Connor *et al.* (2002)
pTO11	*ramR aadA*	pSETΩ	O'Connor *et al.* (2002)
pSETΩ*ramC-tap*	*ramC tap*	pSETΩpcs	This study
pSETΩ*ramCNT-tap*	*ramCNT tap*	pSETΩpcs	This study
pT18	*bld ori colE1 f1 origin T18 MCS^a Amp^r*		Karimova *et al.*, 1998
pT25	*cat ori p15A T25 MCS^a Chl^r*		Karimova *et al.*, 1998
pT18-*ramCNT*	*ramCNT Amp^r*	pT18	This study

[a] MCS, multiple cloning site.

3.0 Results

3.1 RamC accumulation in null mutations of *bldB*, *bldC*, *bldG* and *bldK*

Previous study by O'Connor *et al.* (2002) suggested that *ramC* was active primarily in the substrate hyphae, and its expression is dependent on *bldD*, *cprA* and *ramR*, but not *bldM, bldN, whiA, whiB, whiG, whiH* and *sigF*. To determine whether other well-characterized *bld* mutations altered production of RamC, I compared RamC accumulation in two wildtype strains M145 and J1501 with strains bearing null mutations or defective alleles of *bldB*, *bldC, bldG* and *bldK* (Eccleston *et al.,* 2002; Nodwell *et al.,* 1996; Merrick, 1976; Willey *et al.,* 1993; Champness, 1988). These strains were grown in parallel with their wildtype parent for 24, 36, 48 and 72 hours on cellophane membrane covered rich medium (R2YE), harvested and lysed. Balanced amounts of protein of each lysate (equivalent to the content of 7.5 mg of cells) were separated on SDS polyacrylamide gels. Following electrophoresis, proteins were transferred to PVDF membranes and analyzed by Western blot using affinity purified anti-RamC antibodies (Hudson *et al.*, 2002). The results of the Western analysis are shown in figure 4.

As shown in figure 4, both morphologically wildtype strains (M145 and J1501) exhibited the expected pattern of RamC accumulation and apparent loss. However, M145 produced RamC protein about 12 hours earlier than J1501 and we have no explanation for this. In contrast to wildtype strains, null mutants of *bldC* and *bldK* failed to produce RamC at a detectable level. Defective alleles of *bldB* and *bldG* also caused at least 90% reduction in RamC accumulation. There was some variation in this data: in one version of

this experiment a very low level of RamC was detected in the *bldK* null mutant and none was detected in the *bldG* mutant (data not shown). The Western analysis data of null mutant *bldI* is not shown in figure 4, however, it also fail to produce RamC at detectable level. Nevertheless, these results indicated that *bldB, bldC, bldI, bldG* and *bldK* are all important for the efficient production of the RamC protein. A most surprising observation was that whenever RamC was observed in these *bld* mutants it appeared first at ~24 hours following the initiation of growth then disappeared between 36 and 48 hours, just as it did in wildtype strains. This suggested that the apparent loss of RamC protein observed in wildtype strains might be independent of the formation of aerial hyphae in that it could also occur in mutants blocked in this morphogenetic process.

3.2 RamC accumulation in 138 mutagenesis-generated mutants defective in the formation of aerial hyphae

In order to determine whether the accumulation followed by loss of RamC was a general phenomenon of RamC-producing *bld* mutants and, furthermore, to identify *bld* mutations that altered this pattern, we carried out similar Western analysis on a collection of 138 *bld* mutants isolated through NTG mutagenesis of wildtype strain 1147 (Nodwell *et al.*, 1999). In this screen several hundred *bld* mutants were isolated and 50 were characterized by extracellular complementation analysis with strains containing mutations in *bldJ, bldK, bldA, bldG, bldC* and *bldD* (Nodwell *et al.*, 1999). We have investigated RamC production in 40 of the strains characterized by extracellular complementation analysis and also in a large number of mutants isolated in this screen

that were not previously described in the press (Nodwell, Yang, Kuo and Losick, unpublished observations).

3.2.1 98 mutants that produced a drastically reduced or undetectable level of RamC

In total, we analyzed RamC production in 138 *bld* mutants (not including the *bldB, bldC, bldG* and *bldK* mutants shown above). 98 of these mutations caused a drastically reduced or undetectable level of RamC (Table 2), similar to the strains containing mutations in *bldB, bldK, bldI, bldC, bldG* (this study) *and bldD, cprA* (O'Connor et al., 2002). This included the six mutants previously demonstrated to contain defective alleles of *bldK*: N76 (*bldK203*), N77 (*bldK225*), N78 (*bldK243*), N83 (*bldK557*), N285 (*bldK314*) and N315 (*bld*525)(data not shown) and indeed, all of the mutants previously shown to fit in the K group of the extracellular complementation hierarchy, including all putative alleles of *bldL*. All of the C group mutants N265 (*bld152*), N266 (*bld159*) and N287 (*bld321*) were similarly defective: no RamC production was detected in any of them. The D-like group of mutants, however, were mostly able to produce RamC protein, although in some cases in an altered manner (see below). Of the D-like mutants that we examined, only six, N122 (*bld405*), N124 (*bld170*), N282 (*bld262*), N290 (*bld352*), N319 (*bld533*) and N335 (*bld572*) were defective in production of RamC, as is the *bldD* mutant itself (O'Connor *et al.*, 2002). We did not determine whether these six contained defective alleles of *bldD*. We examined two of the mutants that did not fit the extracellular complementation hierarchy (Nodwell *et al.*, 1998), N312 (*bld512*) and N343 (*bld590*) and both of these were defective in production of RamC. Finally, we identified an additional 78 mutants not characterized by

extracellular complementation that were defective in production of RamC (Table 2). The majority of *bld* mutations therefore, caused a substantial reduction or a complete block in production of RamC, consistent with the results that we obtained with the well-characterized *bld* mutants.

3.2.2 33 mutants that produced RamC in a manner similar to that of their parent

A smaller number of *bld* mutants, 33 in total, appeared to produce RamC at a level and with timing similar to that of their wildtype parent (Table 3). Of the members of this group that had been characterized by extracellular complementation, none were of the K-group, C-group or NF (non-fitting)-group, rather, all had been placed in the D-like extracellular complementation group. Characteristic data is shown for one of these strains, N262 (*bld139*) in figure 5. The data for N262 (*bld139*) was virtually indistinguishable from that of 1147 (Figure 5). We note that some *bld* mutants are partially rescued for production of aerial hyphae when grown on cellophane coated R2YE – the growth conditions that we have employed here. In general however there was no correlation between leakiness during this growth condition and the phenomenon of RamC accumulation and loss. These striking observations strongly suggested therefore, that the formation of aerial hyphae is not required for the precipitous drop in RamC levels that occurs during the growth of *S. coelicolor* colonies.

3.2.3 7 mutants that produced RamC in an altered manner

There was however, a small group of 7 *bld* mutants that produced RamC in an altered manner (Table 4). Figure 6 shows Western analysis of RamC production in strain

N241/*bld61* and N302/*bld429* grown in parallel with the wildtype strain 1147. RamC was evident in N241 (*bld61)* between 24 hours and 36 hours of growth, about 12 hours delay compared to wildtype strain 1147. However, in contrast with the wildtype strains, once RamC protein had become detectable, it remained at a relatively high level for a much longer period of time, and was still present after 72 hours of growth. In contrast to the pattern observed in N241, in the *bld429*-containing strain N302, RamC protein could not be detected until at least 48 hours of growth, a delay similar to that in the *ramR* mutant strain (O'Connor *et al,* 2002). We designated these "delayed and persistent RamC" (D&P) and "delayed RamC" (D) phenotypes. We identified two RamC delayed mutants N239 (*bld38*) and N318 (*bld212*) and three RamC persistent mutants N251 (*bld96*), N292 (*bld361*) and N302 (*bld429*). We also identified two strains in which RamC appearance was both delayed and persistent (D/P): N119 (*bld337*) and N241 (*bld61*).

3.3 No defective alleles of *bldM* and *bldN* found in the mutants that produce RamC

We were most interested in those *bld* mutants that produced RamC protein, particularly those that produced RamC in an altered manner relative to wildtype *S. coelicolor* because the characteristics of the known *bld* gene mutants suggest that these are the most likely to be novel. We know from previous work that mutations in *bldM* and *bldN* do not block RamC production and, furthermore, that these mutant strains may produce a greater amount of RamC protein and that this may persist for a longer time during colony growth (O'Connor *et al.,* 2002). To determine whether any of the 40 RamC producing mutants listed in tables 3 and 4 contained defective alleles of either of these genes, I introduced plasmids pIJ6602 and pIJ6715 (containing cloned *bldM* and

bldN genes respectively) into each of them. The result of this experiment is shown for one mutant N309, in figure 7. While the control vector pSET152 had no effect on the morphogenetic phenotype of any of the strains, we observed the correct pattern of complementation of the *bldM* and *bldN* mutants by pIJ6602 and pIJ6715 respectively. In contrast, the developmental phenotype of N309 was not altered by either plasmid. This was the case for all of the 39 mutants into which we introduced these plasmids. We conclude therefore that these strains contain mutations in other genes that block morphogenesis but not RamC production.

3.4 None of the 7 mutants that produced RamC in an altered manner contained defective alleles of *ramR*

ramR null mutants are defective in the normal timing of RamC production but with prolonged incubation do produce the protein (O'Connor *et al.*, 2002), as did some of the mutants listed in table 4. To determine whether any of the P (persistent RamC), D (delayed RamC) or D&P (delayed and persistent RamC) strains contained defective alleles of *ramR,* we introduced the plasmid pTO11 (O'Connor *et al.*, 2002), which contains a *ramR* gene, into each of them. As shown in figure 8, while this plasmid restored the morphogenetic phenotype of the *ramR* null mutant N376, it had no effect on the formation of aerial hyphae in N251 (*bld*96). Similar results happened to all the other six mutants N119, N241, N292, N239, N302 or N318. Combined with the results mentioned above in 3.3, we believe that these strains all contain mutations in genes other than *ramR, bldM* or *bldN.*

3.5 Potential alleles of *ramC* and *bldC* were identified among the mutants defective in RamC production

As mentioned above, 98 mutants accumulated RamC at a drastically reduced or undetectable level. Many of them might be novel *bld* genes. Identifying new *bld* genes from this group by genetic mapping would be very tedious and it would be preferable to simply complement them *in trans* using a genomic library. As the first in this work, I tried to rule out the strains that contain mutations in *bldC* or *ramC* from this group, since either *bldC* or *ramC* null mutants also produce RamC at undetectable levels. I introduced plasmid pIJ6836 (Buttner lab collection) which contains a cloned *bldC* gene and pSETΩpcs (O'Connor *et al.*, 2002) which contains a *ramC* and *ramS* gene into 18 mutants which had been characterized by extracellular complementation previously by Nodwell (1998). The result has been summarized in table 2. Most mutants would not be complemented by either pSETΩpcs or pIJ6836; the result of one representative mutant N124 is shown in figure 9E.

As shown in figure 9A, while the control vector pSET152 had no effect on the *ramC* mutant N373, pSETΩpcs restored morphogenesis such that a layer of white aerial hyphae was evident on the surface of the mutant colonies. Similarly, plasmid pSETΩpcs, but not the control vector restored the aerial mycelium formation to N282 (*bld*262) (Figure 9C). We conclude therefore that N282 *(bld262)* is a potential defective allele of *ramC*. Strain N282 (*bld*262) has been characterized as a D-like mutant previously (Nodwell *et al.*, 1999). None of the other mutants was complemented by *ramC*.

In addition, the aerial hyphae development in *bld*C mutant was successfully restored by *bldC*-containing plasmid pIJ6836 but not the control vector pSET152 (Figure 9B). Among the 18 mutants, only one (N75 (*bldL173*)) was also induced to develop white fuzzy aerial mycelium by pIJ6836, however, not by control vector pSET152 and pSETΩpcs (Figure 9D). Interestingly, strain N75 (*bldL173*) was identified as a K-like mutant and a member of putative allele of *bldL* in previous study, rather than a C-like mutant (Nodwell *et al.*, 1999).

Most importantly, this work suggests that a large number of these strains must contain mutations in novel genes.

Table 2. *bld* mutants that accumulated a greatly reduced level of RamC 1/3

Strain/mutation	Extracellular Complementation Phenotype	Complementation by pIJ6838 (*bldC*) or pSETΩpcs (*ramC, ramS*)	
N75/*bldL173*	K-like	+	-
N76/*bldK203*	K-like	-	-
N77/*bldK225*	K-like	-	-
N78/*bldK243*	K-like	-	-
N83/*bldK557*	K-like	-	-
N84/*bldL587*	K-like	-	-
N285/*bldK314*	K-like	-	-
N296/*bldL398*	K-like	-	-
N315/*bldK525*	K-like	-	-
N265/*bld152*	C-like	-	-
N266/*bld159*	C-like	-	-
N287/*bld321*	C-like	-	-
N122/*bld405*	D-like	-	-
N124/*bld170*	D-like	-	-
N282/*bld262*	D-like	-	+
N290/*bld352*	D-like	-	-
N319/*bld533*	D-like	-	-
N335/*bld572*	D-like	-	-
N312/*bld512*	NF		
N343/*bld590*	NF		
N72/*bld90*	NT		
N73/*bld97*	NT		
N79/*bld260*	NT		
N80/*bld314*	NT		
N81/*bld398*	NT		
N82/*bld525*	NT		
N227/*bld3*	NT		
N228/*bld4*	NT		
N229/*bld6*	NT		
N231/*bld16*	NT		
N232/*bld12*	NT		
N233/*bld14*	NT		
N234/*bld16*	NT		
N235/*bld18*	NT		

Table 2. *bld* mutants that accumulated a greatly reduced level of RamC 2/3

Strain/mutation	Extracellular complementation phenotype
N237/*bld23*	NT
N238/*bld25*	NT
N243/*bld76*	NT
N244/*bld77*	NT
N245/*bld81*	NT
N247/*bld85*	NT
N248/*bld91*	NT
N249/*bld93*	NT
N250/*bld95*	NT
N252/*bld107*	NT
N253/*bld108*	NT
N254/*bld113*	NT
N255/*bld122*	NT
N256/*bld123*	NT
N258/*bld127*	NT
N260/*bld134*	NT
N261/*bld138*	NT
N263/*bld142*	NT
N267/*bld161*	NT
N268/*bld165*	NT
N269/*bld177*	NT
N270/*bld190*	NT
N271/*bld195*	NT
N272/*bld201*	NT
N275/*bld211*	NT
N276/*bld212*	NT
N277/*bld224*	NT
N278/*bld227*	NT
N279/*bld244*	NT
N280/*bld245*	NT
N281/*bld260*	NT
N283/*bld296*	NT
N286/*bld317*	NT
N288/*bld342*	NT
N289/*bld349*	NT

Table 2. *bld* mutants that accumulated a greatly reduced level of RamC **3/3**

Strain/mutation	Extracellular complementation phenotype
N291/*bld359*	NT
N293/*bld364*	NT
N294/*bld380*	NT
N297/*bld398*	NT
N298/*bld406*	NT
N299/*bld412*	NT
N300/*bld425*	NT
N301/*bld427*	NT
N303/*bld430*	NT
N306/*bld465*	NT
N307/*bld477*	NT
N308/*bld483*	NT
N311/*bld506*	NT
N314/*bld524*	NT
N317/*bld531*	NT
N329/*bld562*	NT
N332/*bld567*	NT
N333/*bld570*	NT
N334/*bld571*	NT
N337/*bld551*	NT
N338/*bld580*	NT
N340/*bld585*	NT
N342/*bld588*	NT
N360/*bldJ23*	NT
N361/*bldJ24*	NT
N362/*bldJ25*	NT
N363/*bldJ27*	NT
N364/*bldJ28*	NT
N365/*bldJ29*	NT

K-like, C-like and D-like – denote extracellular complementation properties identical to those of *bldK, bldC* and *bldD* mutants respectively. NF – denotes mutants that did not fit the extracellular complementation hierarchy. NT – extracellular complementation not tested for this mutant.

Table 3. *bld* mutants having RamC accumulation similar to wildtype strains

Strain/mutation	Extracellular Complementation Phenotype	Complementation by pIJ6602 (*bldM*) or pIJ6715 (*bldN*)	
N88/*bld118*	D-like	-	-
N90/*bld83*	D-like	-	-
N108/*bld315*	D-like	-	-
N114/*bld331*	D-like	-	-
N123/*bld202*	D-like	-	-
N120/*bld304*	D-like	-	-
N127/*bld343*	D-like	-	-
N187/*bld486*	D-like	-	-
N242/*bld73*	D-like	-	-
N257/*bld125*	D-like	-	-
N259/*bld128*	D-like	-	-
N262/*bld139*	D-like	-	-
N274/*bld207*	D-like	-	-
N284/*bld308*	D-like	-	-
N295/*bld382*	D-like	-	-
N322/*bld539*	D-like	-	-
N336/*bld574*	D-like	-	-
N236/*bld20*	NT	-	-
N240/*bld55*	NT	-	-
N246/*bld84*	NT	-	-
N264/*bld143*	NT	-	-
N273/*bld205*	NT	-	-
N304/*bld431*	NT	-	-
N305/*bld462*	NT	-	-
N309/*bld491*	NT	-	-
N310/*bld504*	NT	-	-
N313/*bld516*	NT	-	-
N316/*bld526*	NT	-	-
N325/*bld551*	NT	-	-
N330/*bld563*	NT	-	-
N331/*bld566*	NT	-	-
N339/*bld551*	NT	-	-
N341/*bld586*	NT	-	-

K-like, C-like and D-like – denote extracellular complementation properties identical to those of *bldK, bldC* and *bldD* mutants respectively. NF – denotes mutants that did not fit the extracellular complementation hierarchy. NT – extracellular complementation not tested for this mutant.

Table 4. *bld* mutants altered in timing and/or persistence of RamC

Strain/mutation	RamC production	Extracellular Complementation Phenotype	Complementation by pIJ6602 (*bldM*)	or pIJ6715 (*bldN*)	or pTO11 (*ramR*)
N241/*bld61*	D&P	D-like	-	-	-
N119/*bld337*	D&P	D-like	-	-	-
N292/*bld361*	P	D-like	-	-	-
N251/*bld96*	P	D-like	-	-	-
N239/*bld38*	D	NT	-	-	-
N302/*bld429*	P	NT	-	-	-
N318/*bld212*	D	NT	-	-	-

P – persistent RamC, D – delayed RamC production. K-like, C-like and D-like – denote extracellular complementation properties identical to those of *bldK, bldC* and *bldD* mutants respectively. NT – extracellular complementation not tested.

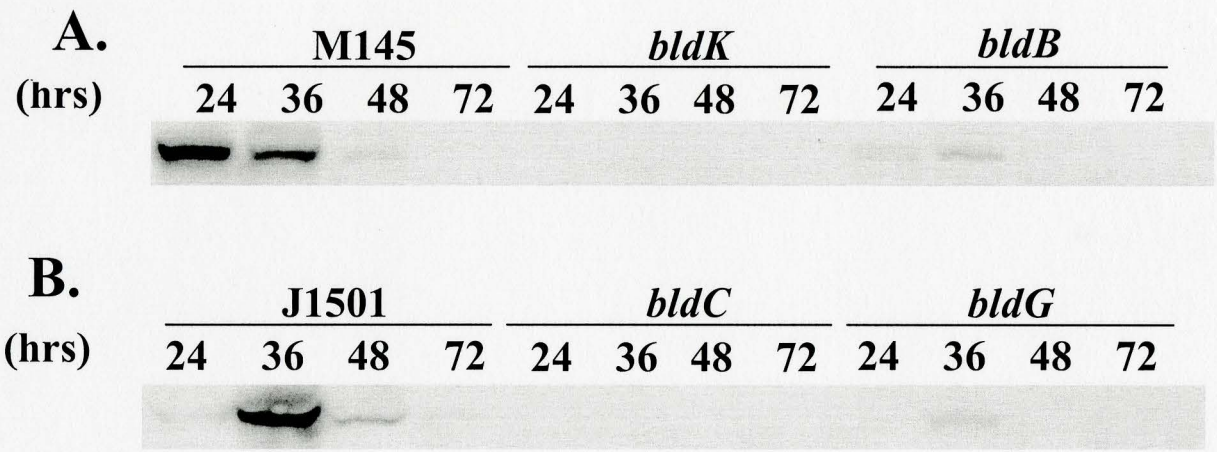


Figure 4. Effects of *bldK, bldB, bldC and bldG* on RamC production. A. Western analysis shows RamC production in null mutation or defective allele in *bldK, bldB* and their parent wildtype strain M145. B. Western analysis shows RamC production in null mutation or defective allele in *bldC, bldG* and their parent wildtype strain J1501. Whole cell lysate (30μl) corresponding to 7.5mg mycelium of each strain was separated on 8% SDS-polyacrylamide gel. The proteins on the SDS-PAGE gel were transferred to PVDF membrane and probed with 500 times diluted affinity purified anti-RamC antibody.

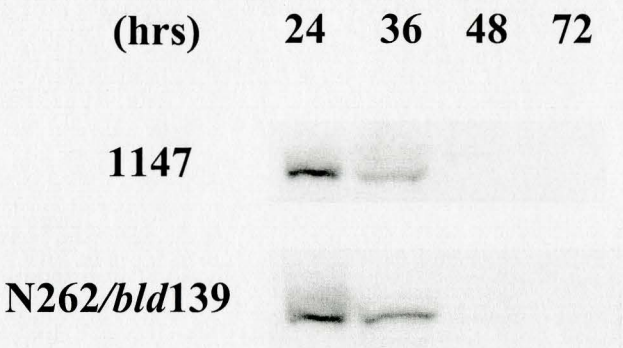


Figure 5. *bld* mutants that produce RamC in similar manner to wildtype *S. coelicolor.* Western analysis shows the time course of RamC accumulation in mutant N262/*bld139* and its parent strain 1147. Whole cell lysate (30μl) corresponding to 7.5mg mycelium of each strain was separated on 8% SDS-polyacrylamide gel. The proteins on the gel were transferred to PVDF membrane and probed with 500 times diluted affinity purified anti-RamC antibody.

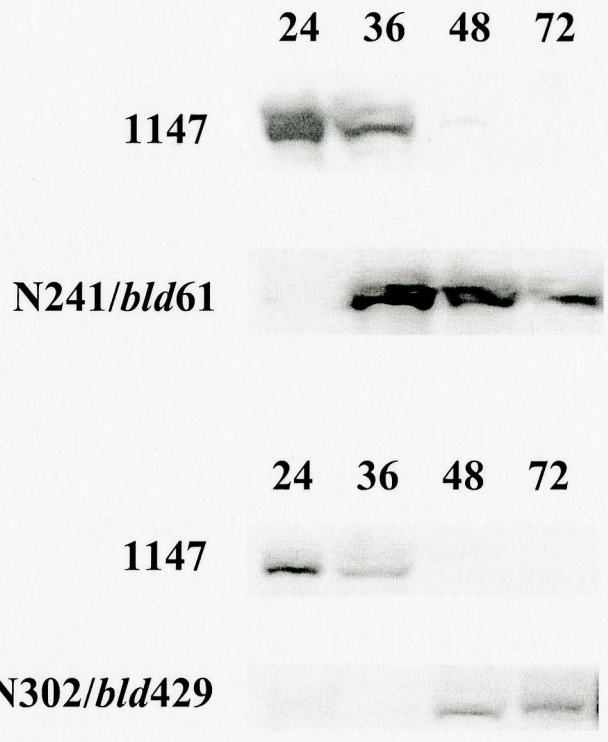


Figure 6. *bld* mutants having altered RamC production. Western analysis shows the RamC accumulation pattern in strains N241/*bld61* and N302/*bld429* in parallel with wildtype strain 1147. Whole cell lysate (30µl) corresponding to 7.5mg mycelium of each strain was separated on 8% SDS-polyacrylamide gel. The proteins on the gel were transferred to PVDF membrane and probed with 500 times diluted affinity purified anti-RamC antibody.

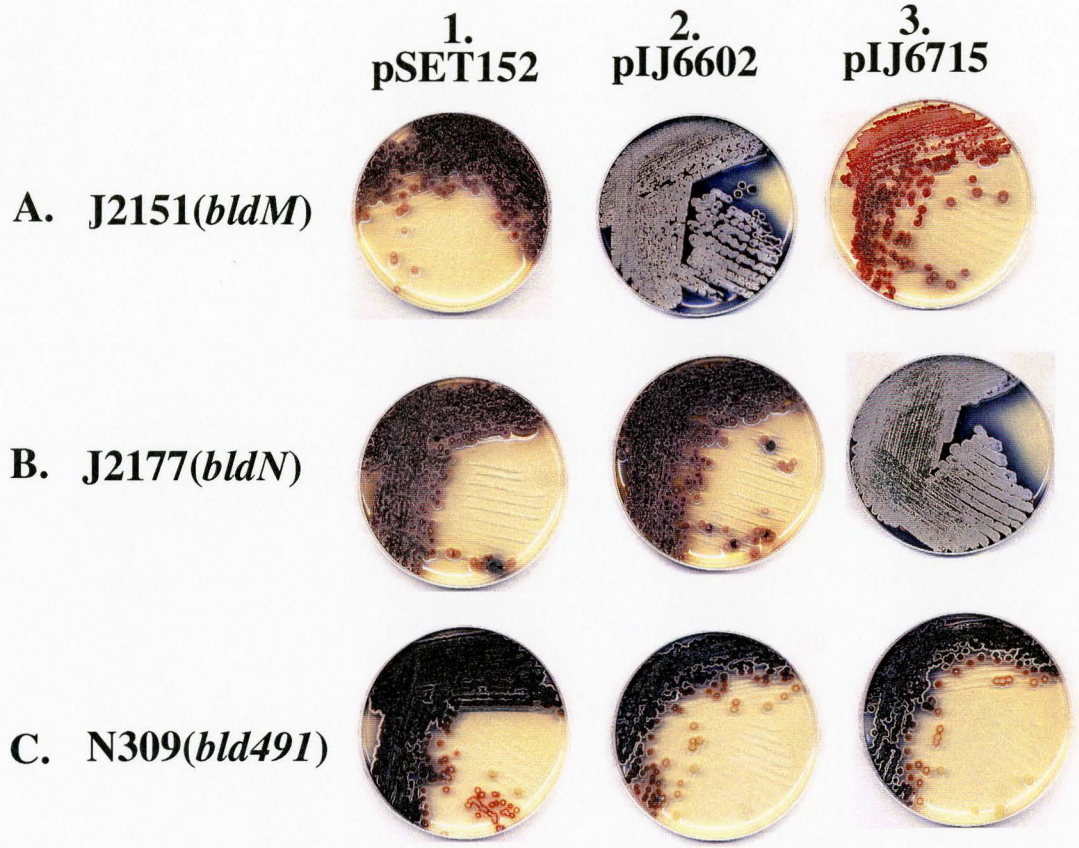


Figure 7. Complementation of RamC producers with *bldM* complementation plasmid pIJ6602 and *bldN* complementation plasmid pIJ6715. Plates show *bldM* null mutant J2151 containing plasmids pSET152 (A1), pIJ6602 (A2) and pIJ6715 (A3); *bldN* null mutant J2177 containing plasmids pSET152 (B1), pIJ6602 (B2) and pIJ6715 (B3); and mutant N309 containing plasmids pSET152 (C1), pIJ6602 (C2) and pIJ6715 (C3). Each strain was restreaked on R2YE plate with no antibiotics and grown at 30°C for 4 days for phenotypic analysis.

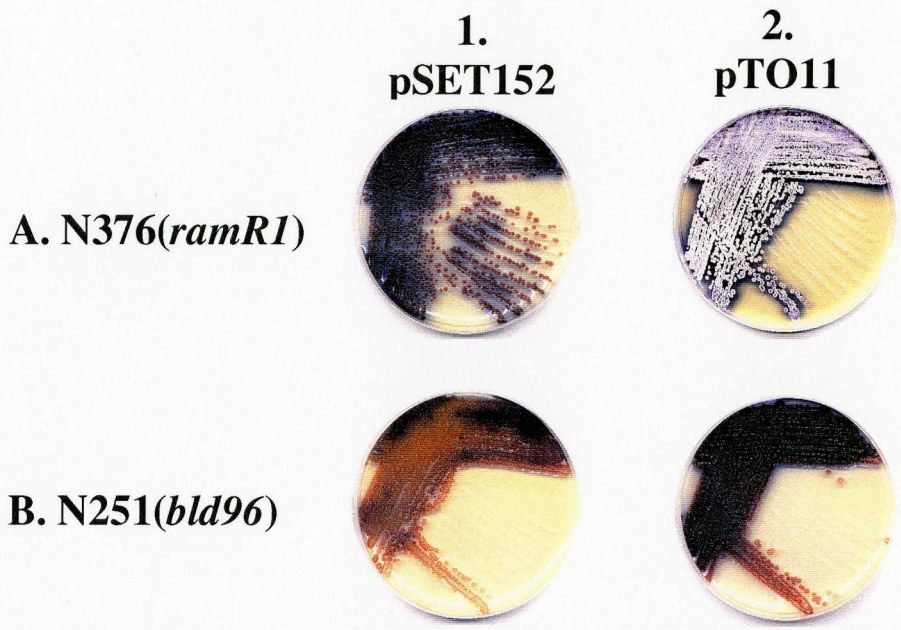


Figure 8. Complementation of *bld* mutants that produced RamC in an altered manner with *ramR* complementation plasmid pTO11. Plates show *ramR* null mutant N376 containing plasmids pSET152 (A1) and pTO11 (A2); and mutant N251 containing plasmids pSET152 (B1) and pIJ6602 (B2). Each strain was restreaked on R2YE plate with no antibiotics and grown at 30°C for 4 days for phenotypic analysis.

Figure 9. Complementation of *bld* mutants that accumulate RamC at a drastically decreased or undetectable level with *ramC* complementation plasmid pSETΩpcs and *bldC* complementation plasmid pIJ6836. Plates show *ramC* null mutant N373 containing plasmids pSET152 (A1), pSETΩpcs (A2) and pIJ6836 (A3); *bldC* null mutant containing plasmids pSET152 (B1), pSETΩpcs (B2) and pIJ6836 (B3); mutant N282 containing plasmids pSET152 (C1), pSETΩpcs (C2) and pIJ6836 (C3); mutant N75 containing plasmids pSET152 (D1), pSETΩpcs (D2) and pIJ6836 (D3); and mutant N124 containing plasmids pSET152 (E1), pSETΩpcs (E2) and pIJ6836 (E3). Each strain was restreaked on R2YE plate with no antibiotics and grown at 30°C for 4 days for phenotypic analysis.

4.0 Discussion

In this work we have confirmed previous observations that suggested that the RamC protein is produced in growing *S. coelicolor* colonies by 24 hours following spore germination, and that production reaches a peak within the next 12 hours and then drops to a level that is too low for detection by Western analysis. This pattern has now been observed repeatedly in the genetic backgrounds M145, J1501 and 1147. Defective alleles of *bldB, bldC, bldG* and *bldK* all caused a dramatic reduction or complete block in RamC production, however, the low levels of RamC production produced by some of these mutants occurred with timing similar to that observed in the wildtype strains. By exploring a large collection of strains containing mutations in unidentified *bld* genes we identified 98 mutants that failed to produce RamC, 33 mutants that produced RamC in a similar manner to wildtype *S. coelicolor* and 7 mutants that produced RamC with altered timing or apparent stability. Using genetic complementation we ruled out the possibility that any of the RamC producing mutants contained defective alleles of *bldM* or *bldN.* Similarly, we showed that none of the 7 strains that produced RamC at an altered manner contained altered alleles of *ramR,* which only produce RamC after a substantial delay. We note that the differences in RamC production in the various *bld* mutants does not reflect a simple difference in strain growth rate because in working with these mutants we have found all but one (N237, containing *bld23,* which grows slowly and did not produce RamC) had essentially identical growth characteristics to their wildtype parent (Nodwell *et al.*, 1999 and data not shown).

Nodwell and Hudson have carried out extracellular complementation analysis of our *ramC* null mutant with the same *bldJ, bldK, bldA, bldG, bldC* and *bldD* mutants used in most previous work (Willey *et al.,* 1993; Nodwell *et al.,* 1996; Nodwell *et al.,* 1999) and found that it did not fit the hierarchy (Nodwell and Hudson, unpublished observations). We found here however that one D-group mutant N282 (*bld262*), was complemented by pSETΩpcs and therefore probably contains a defective *ramC* gene. At present, there is no certain explanation for this discrepancy, and as the *bld262* allele of *ramC* has not been cloned we cannot comment on how it alters RamC protein. Given that we were not able to detect the RamC protein in N282, it may be a nonsense or promoter mutation. Regarding extracellular complementation however, O'Connor and Nodwell note that their evidence suggests that *ramC* null mutation probably exerts a partial polar effect on downstream genes including at least one, *ramS,* that is important for the formation of aerial hyphae by *S. coelicolor* (O'Connor and Nodwell, unpublished observations). We therefore suspect that *bld262* is a non-polar allele of *ramC* and that *ramC* therefore, is a D-group mutant.

There was a fairly strong correlation between the position of mutants in the extracellular complementation hierarchy and their ability to produce RamC. The K group and C group mutants all produced little or no RamC, as did the *bldK, bldG* and *bldC* mutants. In contrast, most of the mutants previously positioned in the D group (excluding *bldD* itself and six of the less well characterized alleles) produced approximately normal amounts of the protein. The fact that most *bld* mutations block RamC production, suggests that the action of the *ramC* gene occurs relatively late in the developmental

pathway that leads to the formation of aerial hyphae. This is consistent with a *ramC* allele falling in the *bldD*-like extracellular complementation group as these mutants are predicted to block morphogenesis at a later stage than the other five groups in the extracellular complementation hierarchy (Willey *et al.,* 1993; Nodwell *et al.,* 1996; Nodwell *et al.,* 1999).

Interestingly, a non-RamC producing strain N75 *(bldL173)* was complemented by plasmid pIJ6836 which contains a *bldC* gene, implying N75 probably bears defective allele of *bld*C. Previous research (Nodwell *et al.*, 1999) suggested N75 exhibited the same pattern of extracellular complementation as defective allele of *bldK*, though not on allele *bld*K, and named as *bld*L. Does the strain N75 really contain the mutation in *bldC*? In order to clarify this question, strain N75 was sent to Buttner lab for further analysis. It turned out that a student in Buttner lab sequenced a *bldC* gene and its promoter region in N75 that was identical to wildtype *bldC*, suggesting N75 does not contain a defective allele of *bldC*. One possible reason to explain that why the wildtype morphogenesis in K-like *bld* mutant N75 (*bldL173*) could be restored by *bldC* containing plasmid pIJ6836 is that having an extra copy of *bldC* bypasses the need for whatever is missing in N75. This is a rare event but not unheard of before. Tamara O'Connor found that Ram (rapid aerial mycelium formation) phenotype could be observed by just having one extra copy of *ramR* in wildtype *S. coelicolor*.

We are most intrigued by the mutants that caused altered RamC production. The RamR protein is a response regulator believed to require phosphorylation in order to carry out its function (Tamara O'Connor and Justin Nodwell, unpublished observations),

however, a cognate sensor kinase has not yet been identified for it at this time. The mutants listed in table 4, therefore, some of which have RamC production characteristics similar to the *ramR* null mutant, therefore, may contain mutant alleles of a gene encoding such a kinase. More importantly, the fact that *bld* mutants can exhibit the same pattern of production and loss of RamC suggests two intriguing hypotheses. First, there may be two distinct populations of substrate hyphae, one of which does not produce RamC and which eventually replaces the other RamC-producing cell type during the *S. coelicolor* life cycle. Alternatively, colonies may possess a mechanism for destroying the RamC protein or the cells that produce it. This second hypothesis may be consistent with observations in other laboratories that the ClpXP protease is important for morphogenesis in *S. coelicolor* (de Crecy-Lagard *et al.*, 1999) or that a mechanism of programmed cell death exists within the substrate mycelium (Miguelez et al., 1999, 2000). We anticipate that the cloning of the genes responsible for the mutant phenotypes of the strains listed in table 4 may shed light on these hypotheses.

Five complementation plasmids containing *bldM, bldN, ramR, bldC* and *ramC* respectively, were introduced into 57 unidentified *bld* mutants by conjugation. Totally 121 conjugations were conducted. Only one mutant was suggested to contain the defective allele of *ramC*. It implies that many novel *bld* genes exist in these 138 unidentified *bld* mutants. Further complementation of these *bld* mutants with chromosomal library of *S. coelicolor* might facilitate the identification of new *bld* genes.

5.0 APPENDIX: Technology for identifying protein partners of RamC

The RamC protein is believed to be a membrane associated serine/threonine kinase (Hudson *et al.*, 2002). The primary goal of this work was to identify proteins that interact with RamC amino terminus, including if possible, potential kinase targets. A genetic approach (bacterial two-hybrid system) and a biochemical approach (tandem affinity purification - TAP) were performed for this purpose. Unfortunately, no convincing RamC partners were identified in the two-hybrid screen and the TAP tagging approach turned out to be excessively difficult. Nevertheless, some interesting observations were made using this approach. Finally, we conducted chemical cross-linking of RamC in wildtype strain M145 and on RamC expressed in *E. coli*. The preliminary results suggest a possible oligomeric state exists, and furthermore, a crosslinked product implying one or more binding partners of RamC. However, further investigation need to clarify these findings.

5.1 Methods

5.1.1 *S. coelicolor* Chromosomal Library Screening

Bordetella pertussis produces an adenylate cyclase encoded by *cyaA* gene. The catalytic domain of the adenylate cyclase is located in the first 400 amino acids, which can be cleaved into two complementary fragments, amino terminal fragment T25 (amino acids 1-224) and carboxyl terminal fragment T18 (amino acids 225-399) (Figure 11A). Plasmids pT25 (Cm^R) and pT18 (Amp^R) were constructed by Karimova *et al.* (1998) to

express protein fragment T25 and T18 respectively. The portion of *ramC* encoding the RamC kinase domain and an *S. coelicolor* chromosomal DNA library have been constructed into plasmids pT18 and pT25 respectively to produce pT18-*ramC*NT and pT25-CL by a previous lab member and Nancy Sheeler. The plasmid pT18-*ramC*NT was introduced into DHP1, an endogenous adenylate cyclase deficient *E. coli* strain. Plasmids pT25-CL were then electroporated into electroporation-competent DHP1 cells containing pT18-*ramC*NT, and plated on MacConkey agar plates supplemented with 1% maltose. After 48 hours growth at 30°C, all red colonies were picked and restreaked onto new MacConkey agar plates supplemented with 1% maltose. If the colonies remained red, the DNA was purified, introduced into *E. coli* XL1-blue cells and plated on LB agar plus 30μg/ml chloramphenicol, permitting growth of the transformants that harbor the plasmid pT25-CL (Chloramphenicol resistant) from the chromosomal library and separating it from pT18-*ramC*NT. Plasmid pT25-CL was then purified from the chloramphenicol resistant colony, transformed back into DHP1 cells containing pT18-*ram*CNT and then plated on MacConkey agar plate supplemented with 1% maltose to confirm the positive interaction again. If all of the colonies on MacConkey agar plates supplemented with 1% maltose were red, the specific plasmid pT25-CL was sent for sequencing.

5.1.2 The recombination of *tap* cassette to the carboxyl terminal of full length *ramC* and the amino terminus *ramC*

The *tap* cassette (Cm^R) that was used for tagging full length *ramC* was amplified from plasmid pJh71 (cut once with *Apa*I) with primer 5'-CGCCGCCGCGCCGAAGCGG CGGCCCCCTGACTCGGCCCCACCAGGAGCCGTCCATGGAAAAGAGAAG and

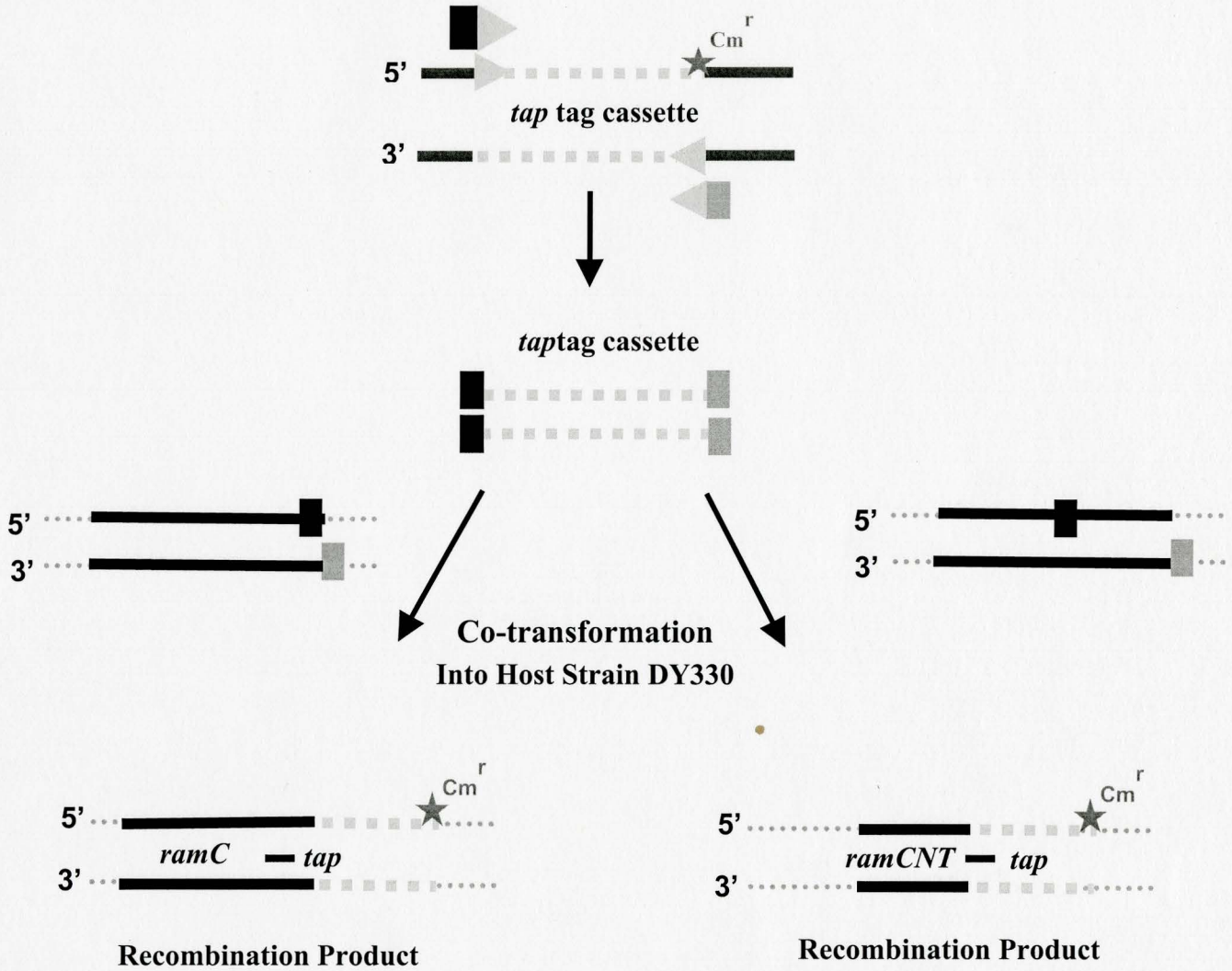


Figure 10. Principle of λ recombination system. The *tap* cassette was amplified from plasmid pJh71 with a pair of primers that contain two parts, a 3'end that primes the *tap* cassette DNA for amplification and a 5' end homologous to flanking sequence of the target DNA fragment for replacement. Purified *tap* cassette is introduced into competent λ recombinase expressing DY330 cells along with target plasmid for the recombination. The correct recombinants could be selected by growing on LB agar plates plus 30μg/ml chloramphenicol.

primer 5'-CGAACAGGTTCATGACGCATTCCTTTCGTTCGGCAAGGGGTGTGCTT TTCCATATGAATATCCTCCTTAG. The *tap* cassettes (Cm^R) that was used for tagging the amino terminal *ramC* was amplified from plasmid pJh71 with primer 5'-CGAACCCGGGGTTCGTGGCGCCTCCCGACCGGCGCGGGGTGGCCGTGGACTC CATGGAAAAGAGAAG and primer 5'-CGAACAGGTTCATGACGCATTCCTTTC GTTCGGCAAGGGGTGTGCTT TTCCATATGAATATCCTCCTTAG. All the primers contain two parts, a 5' end of about 50 bases which is homologous to flanking sequence of the target DNA fragment for replacement and a 3' end of about 20 bases that primes the *tap* cassette DNA for amplification. Both PCR purified *tap* cassettes were about 1.6kb.

λ recombination system was used for tagging full length *ramC* and the amino terminus *ram*C with the *tap* cassette (Figure 10). λ recombinase expressing strain DY330 was used as the host strain. The *tap* cassette (about 100ng) was electroporated into freshly prepared electroporation-competent DY330 cells (Yu *et al.*, 2000) with target plasmid pSETΩpcs ($Spec^R$) (Tamara O'Connor) and plated on LB agar plus 30μg/ml chloramphenicol and 100μg/ml spectinomycin to select the correct recombinants. The recombination of the *tap* cassette to both full-length *ramC* (pSETΩ*ram*C-*tap*) and the amino terminus *ramC* (pSETΩ*ramCNT-tap*) were further confirmed by restriction enzyme analysis and sequencing results.

5.1.3 Western Analysis of RamC-TAP and RamCNT-TAP expression in *S. coelicolor*

Plasmids pSETΩ*ramC-tap* and pSETΩ*ramCNT-tap* were purified from methylation deficient strain Er^2-1 and introduced into *S. coelicolor* wildtype strain M145 and *ramC* null mutant strain N373 by protoplast fusion respectively (Kieser *et al.*, 2000). Each strain was grown on porous cellophane membrane covered R2YE plates at 30°C for 40 hours. The cells were then harvested by scratching the mycelium off the cellophane membrane and weighed. After washing twice with 1ml saline each time, the cells were spun down at 18,000 ×g for 20 minutes. The supernatant was decanted and the cells were resuspended in 800µl saline containing 0.5mg/ml lysozyme and incubated at room temperature for 15 minutes. About 400µl 3 fold SDS sample buffer were added to each sample thereafter and the samples were boiled for 15 minutes. The cell debris was separated from the whole cell lysate by spinning at 18,000 ×g for 10 minutes and discarded. The supernatant, which contained the whole cell lysate, was transferred to a new tube for Western analysis.

The whole cell lysate of each strain, corresponding to 2mg cells, were diluted to 30µl with 1 fold SDS sample buffer and fractionated on 8% SDS-polyacrylamide gel. Two identical SDS-polycrylamide gels were run at the same time. The proteins on both gels were transferred to two polyvinylidene difluoride (PVDF) membranes under the same condition at the same time. One membrane was probed with affinity purified anti-RamC antibody (Hudson *et al.*, 2002) followed by the HRP coupled anti-rabbit secondary

antibody (Amersham Pharmacia Biotech). The other one was probed with HRP coupled anti-rabbit secondary antibody only.

5.1.4 Preparation of solubilized expressing cell extract

Membrane fraction of M145+pSETΩ*ramC-tap* and M145+pSETΩ*ramCNT-tap* were prepared as previously described for M145 (Hudson, *et al.,* 2002). The membrane pellets that were obtained after a one-hour ultracentrifugation at 100,000×g were resuspended in the chilled solubilizaion buffer (80mM Na_2HPO_4, 20mM NaH_2PO_4, 100mM NaCl, 1mM DTT, 1mM EDTA, 1 fold protease inhibitors cocktail, pH7.0). The concentration of each membrane fraction was determined by Bradford Assay and then adjusted to the final concentration of 15mg protein per ml. The membrane fractions were stored at -20°C as stock membrane fraction. Before performing the detergent mediated solubilization, the stock membrane fraction was freshly thawed and diluted to 1 – 5 mg protein /ml with chilled solubilization buffer. The DHPC (Avantis) stock solution (200mM, stored at 4°C) was then added to an ideal final testing concentration. The samples were vortexed briefly to mix well and incubated on ice for 30 minutes. After the treatment with DHPC, the soluble membrane proteins were extracted from insoluble proteins by ultracentrifugation at 100,000×g for 30 minutes. The supernatant that contains the soluble membrane proteins was transferred to a new tube. An equal volume of solubilization buffer as supernatant was added to resuspend the pellet for further analysis of the insoluble proteins that were left in the pellet. Half volume of 3 times SDS

sample buffer was added to the supernatant and pellet suspension and the samples were boiled for 15 minutes for Western analysis against anti-RamC antibody.

5.1.5 Covalent cross-linking of RamC

Wildtype strain M145 and *ramC* null mutant N373 were plated on cellophane covered R2YE plates. After grown at 30°C for 36 hours, 200 milligram mycelium of each strain was harvested and resuspended in 1ml P-buffer containing 2mg/ml lysozyme. After incubation at 30°C for 2 hours, the samples were aliquoted to eppendorf tubes with 250µl suspension (~50mg cells) in each. The aliquoted samples were then washed twice with 200µl crosslinking buffer (100mM HEPES, 100mM NaCl, 10% Glycerol, pH 9.0) each time to get rid of any primary amine in the P-buffer. After centrifugation at 18,000 ×g for 5 minutes, the supernatant was decanted and the cell pellets were resuspended in 180µl crosslinking buffer containing no DMS, 10mM DMS, 20mM DMS and 40mM DMS. All DMS solutions were freshly prepared just before crosslinking experiment. The cells were incubated with DMS for 1 hour at room temperature with occasional vortex. 20µl 1M Tris buffer was added to all the samples to stop the reaction. The samples were then mixed with 100µl 3 times SDS sample buffer and boiled for 15 minutes. Cell debris was spun down by a 1minute centrifugation at 18,000 ×g and discarded. The supernatant that contains the whole cell lysate was transferred to a new tube for western analysis.

The above crosslinking procedure was scaled down to be performed on the membrane fraction of *E. coli* strains BL21- (DE3)-plysS containing *ramC* expressing plasmid pEMH-1 and background vector pE21a respectively, which were kindly donated

from Mike Hudson. The original concentration of the membrane fraction was 5.25mg/ml. Since the storage buffer (100mM HEPES, 2mM DTT, 2mM EDTA, 100mM NaCl, 10% Glycerol, pH 7.5) of the membrane fraction does not contain primary amine, no washing step was needed here before crosslinking. 3μl membrane fraction (~15mg proteins) was added to 33μl crosslinking buffer with no DMS, 20mM DMS and 40mM DMS respectively. 4μl of 1M Tris buffer was added to stop the reaction after 1 hour. Finally, 7μl of 3 times SDS sample buffer was added to each sample and boiled for 15 minutes. As above, the supernatant that contains the whole cell lysate was transferred to a new tube for Western analysis.

Whole cell lysate (20μl) of each sample was separated on 8% SDS-PAGE. The proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore) and probed with affinity purified anti-RamC antibody.

5.2 Results

5.2.1 *Streptomyces coelicolor* chromosomal library screening

In order to identify genes encoding RamC binding partners, I used bacteria two-hybrid system to screen a chromosomal library of *S. coelicolor*. Two-hybrid system allows for the detection of weak and transient interactions since the genetic reporter strategy results in a significant amplification (Criekinge *et al.,* 1999). Furthermore, one of the most appealing features of the two-hybrid system is that at the same time of identifying an interacting protein, the corresponding gene is cloned.

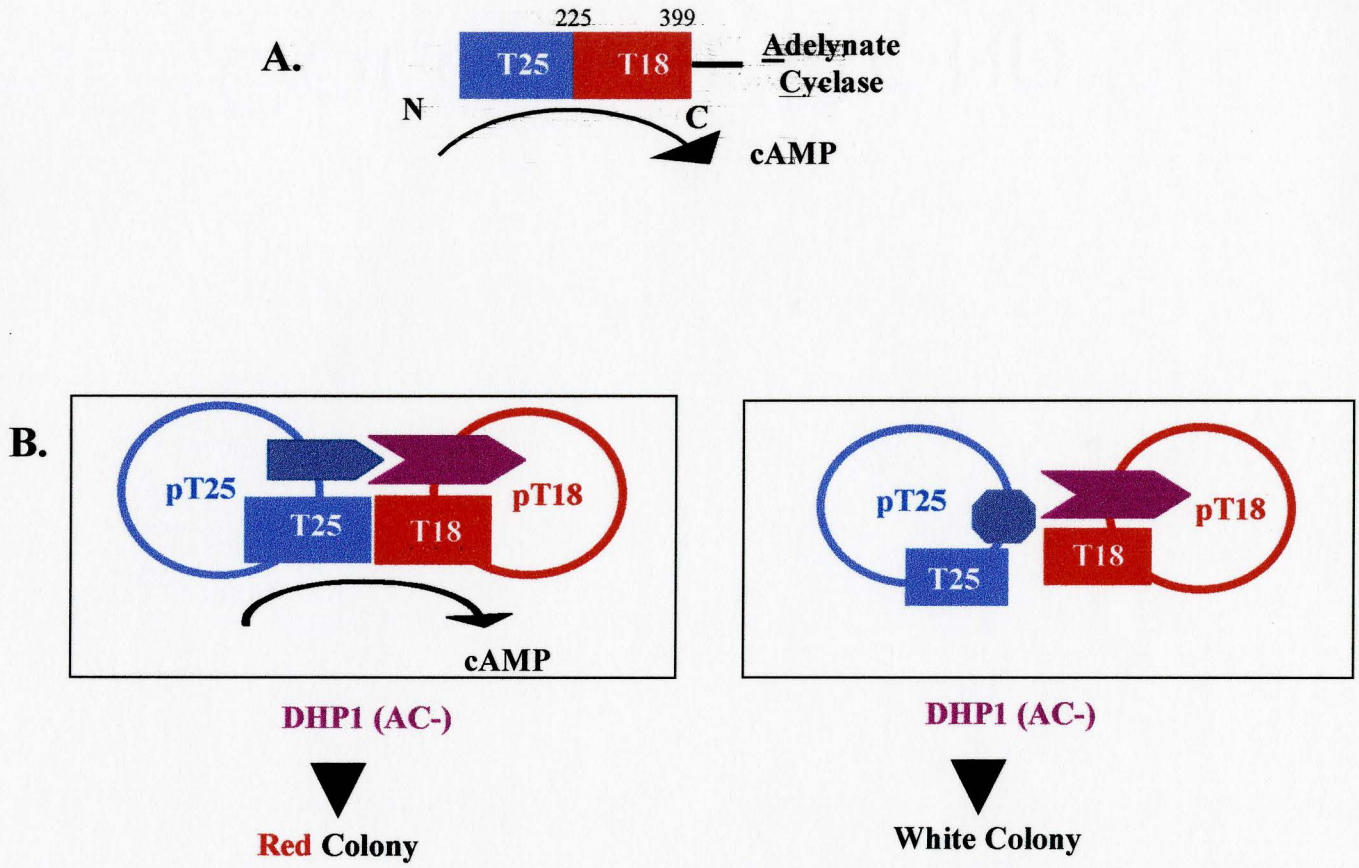


Figure 11. Principle of an *E. coli* two-hybrid system based on functional complementation of adenylate cyclase. A. The catalytic domain (residue 1-399) of *Bordetella pertussis* adenylate cyclase is essential for the cAMP synthesis. The blue box and red box represent the T25 and T18 fragments corresponding to amino acids 1 – 224 and 225 – 399 respectively. B. T25 and T18 fragments are constructed into two different plasmids pT25 and pT18. Either one cannot induce cAMP production in an adelynate cyclase deficient strain DHP1 by itself. Two putative interacting genes are fused into pT25 and pT18 respectively. When two plasmids are co-expressed in DHP1, the cAMP synthesis can be restored through the interaction of these two interacting genes, which bring a functional complementation between fragments T25 and T18. The production of cAMP therefore induces maltose fermentation on indicator plates (MacConkey agar plates supplemented with 1% maltose) and red colonies are developed. In contrast, white colonies indicate no cAMP production.

Recent research by Hudson revealed that the amino terminal half of the RamC contains a protein kinase-like motif that is composed of up to 9 Hanks domains (Figure 3B), and those were shown to be essential for RamC's function in *vivo* (Hudson *et al.*, 2002). Moreover, the full-length RamC is quite big (97 kDa) and is membrane associated. Therefore, the amino terminal half of RamC (46 kDa) containing only the putative kinase domain was chosen as the target in this screening.

The bacterial two-hybrid system I used in this work is based on the reconstitution of adenylate cyclase in an *E. coli cya* strain DHP1. The catalytic domain of the adenylate cyclase in *B. pertussis* can be cleaved into two complementation fragments, the amino terminal fragment T25 (amino acids 1-224) and carboxyl terminal fragment T18 (amino acids 225-399) (Figure 11A). Genes encoding the two fragments were cloned into two plasmids and named as pT25 (Cm^r) and pT18 (Amp^r) respectively by Karimova *et al.* (1998). A previous lab member constructed a fusion of the portion of *ramC* encoding the RamC kinase domain into plasmid pT18 to produce pT18-*ram*CNT. Nancy Sheeler constructed a library of *S. coelicolor* chromosomal DNA in plasmid pT25 consisting of ~ 90,000 independent clones (Sheeler & Nodwell unpublished observation). They were referred to as pT25-CL in general. When pT18-*ramCNT* and pT25-CL are co-expressed in an adelynate cyclase deficient *E. coli* strain DHP1, if a chromosomal DNA fragment on pT25-CL encodes a protein or protein fragment that interacts with RamC amino terminal half, protein fragment T18 and T25 would reassociate and lead to reconstitution of the catalytic domain of adenylate cyclase. As a result, the adenylate cyclase-deficient *E. coli* strain DHP1 regains its ability to produce cAMP, which activates maltose

fermentation in MacConkey agar plates supplemented with 1% maltose and red colonies are developed, whereas the white colony implies no protein-protein interaction (Karimova *et al.*, 1998) (Figure 11B).

The chromosomal library consists of 90,000 – 120,000 distinct clones of between 1.5 and 6kb. In the process of screening I examined about 200,000 colonies. The result was listed in table 5.

112 positive candidates were initially identified. As mentioned before, one advantage of two-hybrid system is its detection of weak interactions, however, one weakness of this system is that false positive results may be encountered when performing a screening procedure. Therefore, in order to rule out false positive candidates, the specific candidate plasmid pT25-CL was purified from red colonies and its interaction with pT18-*ramCNT* was tested again by introducing it back into competent DHP1 cells containing pT18-*ramCNT*. Only three candidates remained positive and gave rise to red colonies. The sequencing of these three candidates revealed that none of them was of interest to us (Table 5). Chromosomal DNA fragment 30-1 encodes part of the adenylate cyclase of *S. coelicolor*. It was capable of inducing maltose fermentation only when it interacted with pT18-*ram*CNT, but not by itself. In order to clarify whether the positive interaction was actually due to its interaction with RamC or with the protein fragment T18 of adenylate cyclase of *B. pertussis*, I transformed the candidate plasmid pT25-30-1 into DHP1 cells containing a fusion of a different gene, *bldB*, to pT18. All the colonies on the indicator plate were red, indicating that the protein fragment of the *S. coelicolor* adenylate cyclase encoded by DNA fragment 30-1 bound to the T18 fragment

of *B. pertussis* adenylate cyclase rather than RamCNT. The DHP1 strains containing pT25-30-1, pT25-30-1 + pT18-*ramCNT* and pT25-30-1 + pT18-*bldB* were restreaked and compared on one MacConkey agar plate plus 1% maltose and shown in figure 12. The other two candidate DNA fragments 5-1 and 18-1 showed almost the identical sequence alignment and seemed to be a recombination of T18 DNA fragment into plasmid pT25, which resulted in the generation of a functional adelynate cyclase catalytic domain in DHP1. These candidates could induce the maltose fermentation in the absence of pT18-*ramCNT*. The reason of the recombination is not clear.

We are fairly confident that our two-hybrid library contains a good representation of the *S. coelicolor* genome because it has been used successfully in another project. There are two reasons we proposed to be related to the failure to pull down any partner of RamC. First of all, the use of fusion protein always has a potential risk. The fusion might change the actual conformation of proteins, block the accessibility of binding sites and consequently alter its functionality. Amino terminal RamC might be inappropriately folded and make it difficult to bind to other proteins. Moreover, it is also possible that two putative interacting proteins, although exist and able to interact, are never in close proximity to each other within the DHP1 cells due to different localization and expression time points (Criekinge *et al.*, 1999). Finally, we decided to abandon this approach.

Table 5. Summary of screening the interacting protein of RamC from a chromosomal library of *S. coelicolor* using a bacteria two-hybrid system. (* CL – chromosomal library, Red – denotes red colonies that caused by positive protein-protein interaction, White – denotes white colonies that indicate no protein- protein interaction)

Positive colonies from the original transformation	**Candidates sent for sequencing**		
112	3		
	5-1	18-1	30-1
pT18-*ramCNT* + pT25-CL* in DHP1	Red	Red	Red
pT25-CL* in DHP1	Red	Red	White
Results of sequencing and database search	T18 fragment recombined into pT25-CL plasmid	T18 fragment recombined into pT25-CL plasmid	DNA sequence that encodes part of adenylate cyclase of *S. coelicolor*

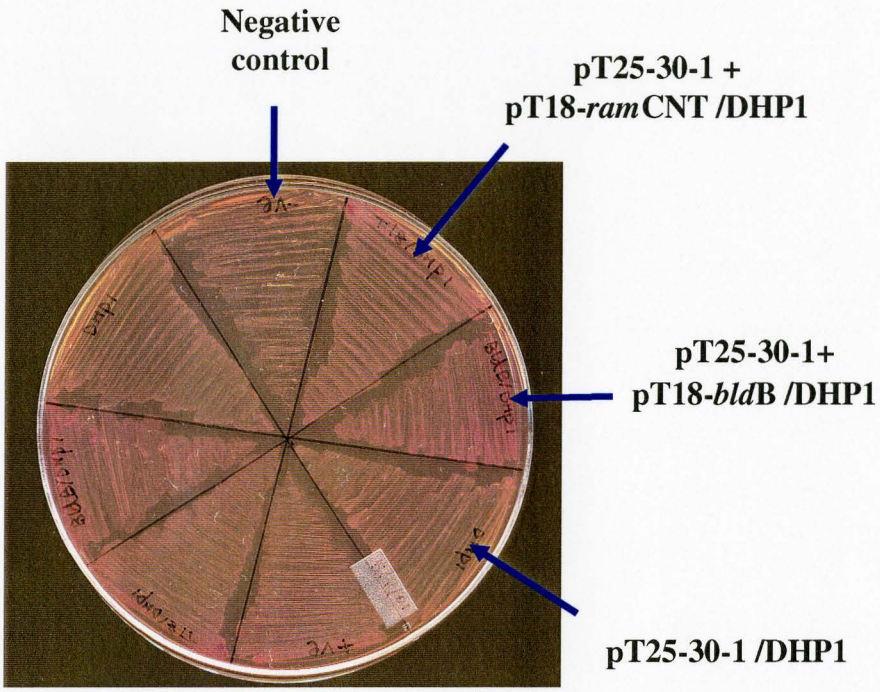


Figure 12. The positive interactions between plasmid pT25 which contains candidate clone 30-1 and pT18-*ramC*NT and pT18-*bldB*. DHP1 cells containing pT25-30-1, pT25-30-1 and pT18-*ram*CNT, pT25-30-1 and pT18-*bldB* were restreaked on MacConkey agar plate plus 1% maltose and grown for 48 hours. Red colonies in pT25-30-1 + pT18-*ram*CNT and pT25-30-1 + pT18-*bldB* indicate maltose fermentation caused by positive protein-protein interaction, whereas white colonies in pT25-30-1 indicate no protein-protein interaction.

5.2.2 Tap-Tag mediated complex purification

The tandem affinity purification (TAP) has been widely used recently to identify interactions existing among gene products (Puig *et al.,* 2001, Rigaut *et al.,* 1999). We attempted to use Tap-tag sequential affinity purification to purify RamC and hopefully, associated proteins under native conditions.

The TAP (tandem affinity purification) tag fragment protein, developed by Rigaut *et al.* (1999), contains two IgG binding domains of Protein A (ProtA) and a calmodulin binding peptide (CBP) separated by a TEV protease cleavage site (Figure 13B). The TAP cassette also contains a chloramphenicol resistant marker. The *tap* cassette firstly needs to be attached to a target gene and introduced into host cells. The cell lysate that express the TAP tagged protein should be prepared and used for TAP mediated purification. Purified protein complex could be fractionated on SDS-PAGE, identified by mass spectrometry and also used for further functional study (Figure 13A).

The TAP mediated purification includes two consecutive affinity purifications. The cell extracts are first loaded onto an IgG affinity column. Protein A binds to the IgG matrix tightly, requiring the use of the TEV protease to elute binding protein complex under native conditions. The eluate from the IgG matrix is then incubated with calmodulin resin in the presence of calcium. After washing off the TEV proteases and other contaminants, the bound TAP tagged protein is released with EGTA (Figure 13C) (Rigaut *et al.,* 1999).

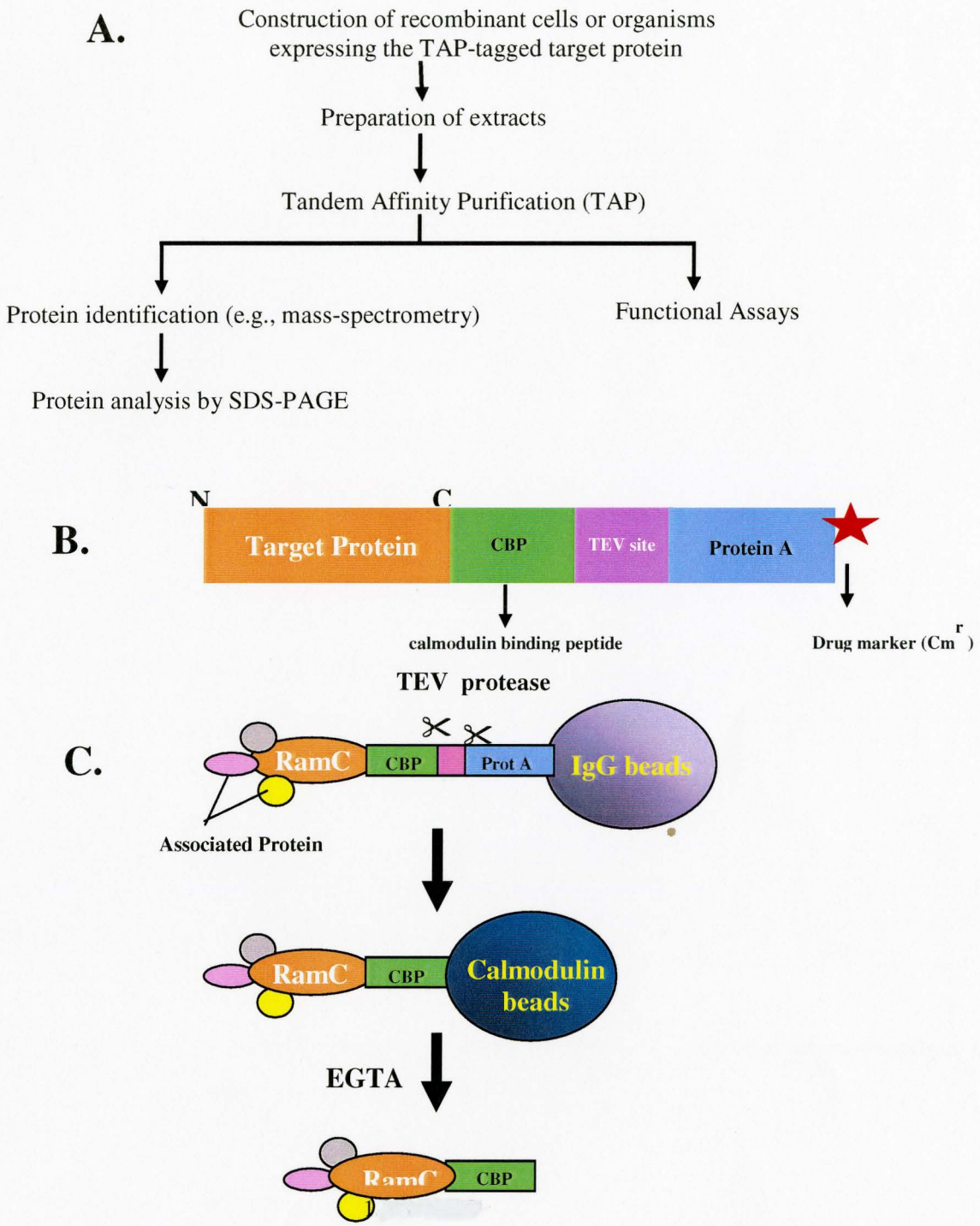


Figure 13. The overview of TAP purification strategy. A. The general procedures of TAP strategy. (www.embl-idelberg.de /ExternalInfo /seraphin / overview.gif) B. The components of TAP tag. C. The general procedures of TAP mediated affinity purification.

In this work, the *tap* cassette was fused to the carboxyl terminal end of both full-length *ramC* and the amino terminus *ramC* in plasmid pSETΩpcs to produce pSETΩ*ramC-tap* and pSETΩ*ramCNT-tap* by using λ recombination system (Figure 10) (Yu *et al.,* 2000). The attachment of *tap* cassette allows the expression of protein RamC and RamC N-terminus under the control of *ramC*'s natural promoter.

Plasmids pSETΩ*ramC-tap* and pSETΩ*ramCNT-tap*, along with the background plasmid pSETΩpcs, were passed through methylation deficient *E. coli* strain Er^2-1 and introduced into *S. coelicolor* wildtype strain M145 and *ram*C null mutant N373 respectively. As shown in figure 14, pSETΩ*ramC-tap* successfully complemented *ram*C null mutant N373 to a similar level as N373 + pSETΩpcs, and gave rise to white aerial hyphae, indicating that RamC did not lose its function when TAP tag is attached. However, pSETΩ*ramCNT-tap* was unable to complement *ram*C null mutant N373. Since we do not know yet whether gene *ramCNT* itself is capable of complementing *ram*C null mutant N373 or not, we cannot simply evaluate the effect of the TAP tag attachment on the RamCNT by this experiment.

Furthermore, the expression level of the TAP tagged proteins was checked by Western analysis. The whole cell lysate of M145+pSETΩ*ramC-tap,* N373+pSETΩ*ramC-tap*, M145+pSETΩ*ramCNT-tap* and N373+ pSETΩ*ramCNT-tap,* corresponding to 2mg cells, were fractionated on 8% SDS-PAGE gel for Western analysis. In theory, the native RamC, TAP tagged RamC and TAP tagged RamCNT, if expressed with no problem, should all be able to be detected by anti-RamC antibody, whereas TAP tagged RamCFL

(RamC-TAP) and TAP tagged RamCNT (RamCNT-TAP) can also be detected by secondary antibody only, as the TAP tag contains two IgG binding domains. The Western results were perfectly consistent with our hypothesis (Figure 15). By probing with secondary antibody only (Figure 15A), the bands of predicted sizes of RamC-TAP (~117 kDa) and RamCNT-TAP (~67 kDa) could be detected in both host strains: wildtype M145 and *ramC* null mutant N373. In contrast, when probed with anti-RamC antibody followed by secondary antibody (Figure 15B), besides the signals believed to be protein RamC-TAP and RamCNT-TAP that appeared in the panel A, native RamC protein (~97 kDa) could be detected in all the wildtype M145 host strains, including M145+pSETΩ*ramC-tap* and M145+pSETΩ*ramCNT-tap,* but absent in the strains N373+pSETΩ*ramC-tap* and N373+ pSETΩ*ramCNT-tap*. These results suggested that RamC-TAP and RamCNT-TAP were expressed with no problem under the control of the natural promoter of *ramC*.

Previous study (Hudson *et al.,* 2002) suggested that RamC was a membrane-associated receptor kinase. Using the same membrane fraction preparation procedure (Hudson *et al.,* 2002), I found the TAP tagged RamC and RamCNT were also located in the membrane fraction (Figure 16). Therefore, before the TAP purification, the solubilized cell extracts need to be prepared under mild condition to maintain the activity of TAP tagged proteins.

A short-chain phosphatidylcholine DHPC (1,2- Diheptanoyl-sn-Glycero-3-Phosphocholine) was our first choice of a mild detergent. Some published studies (Kessi

et al., 1994, Schivanna *et al.*, 1997, Potter *et al.*, 2000) have shown that DHPC was successful in solubilizing a variety of membrane proteins, while retaining native protein structure and activity. Furthermore, DHPC is stable over a wide pH range of 4 – 10, and it does not interfere with spectrophotometric measurements, which is ideal for further affinity purification.

The concentration range of DHPC 5 – 30 mM was tested on the membrane fraction with concentration from 1mg/ml to 5mg/ml. The overall recovery of soluble RamC-TAP and RamCNT-TAP was very poor. The best recovery for both was below 5%. The Western analysis of the best recovery of soluble RamC-TAP from membrane fraction was shown in the figure 18A. Obviously, the recovery was too low to give enough protein for TAP purification.

Some other detergents (Figure 17), including Triton X-100, CHAPS (Sigma) and Fos-choline series (Anatrace), were then applied to solubilize RamC-TAP protein complex from the membrane fraction of the strain M145+pSETΩ*ramC-tap* by using the same protocol. The results of these detergents mediated solubilization of ramC-TAP from membrane fraction are summarized in the table 6. The best recovery of soluble RamC-TAP seemed to be about 40% by using Fos-choline 10 and 50% by using Fos-choline 12. As shown in figure 18, when compared to the insoluble RamC-TAP, only about 5% protein RamC-TAP could be solubilized by DHPC and 10% by Triton X-100. However, with the treatment of Fos-choline 12, the signal of soluble RamC-TAP, even though still weak, seemed to be equivalent to that of insoluble RamC-TAP (Figure 18C). According to the experience in Dr. Andrew's lab (McMaster University, personal communication),

in the presence of Fos-choline 12, the proteins tend to float at the surface of the sample and not get into the SDS-PAGE gel. Therefore, we proposed that Fos-choline 12 was a more efficient detergent for RamC-TAP solubilization than other detergents used, and Fos-choline 12 was used to solubilize large amount of cell extract for a preliminary TAP purificaion.

Totally 12.25 gram mycelium of M145+pSETΩramC-tap was harvested after 40 hours growth. The membrane fraction was prepared and then subjected to solubilization by using fos-choline 12. One fourth of the cell extracts, about 10 millilitres, was used for TAP purification. Following the procedures (Rigaut *et al.*, 1999), the final elution from calmodulin column was analyzed on 8% SDS-PAGE along with flow-through fraction from IgG affinity column, wash-off fraction from IgG column, elution from IgG column and flow-through fraction from calmodulin column. However, no bands of any size between RamC (97 kDa) and RamC-TAP (117 kDa) could be detected in all these fractions (data not shown), no matter in Western analysis or silver stained SDS-PAGE gel. Therefore, it seemed that the problems in the solubilization experiment seriously blocked the further purification.

Finally, we gave up on this approach in favor of a chemical cross-linking experiment.

Figure 14. Complementation of *ramC* null mutant N373 by pSETΩpcs, pSETΩ*ramC-tap* and pSETΩ*ramCNT-tap*. pSETΩ*ramC-tap* induced arerial mycelium formation in *ramC* null mutant N373 to a similar level as *ramC* complementation plasmid pSETΩpcs, whereas pSETΩ*ramCNT-tap* was unable to complement N373. Each strain was restreaked on R2YE plate with no antibiotics and grown at 30°C for 4 days for phenotypic analysis.

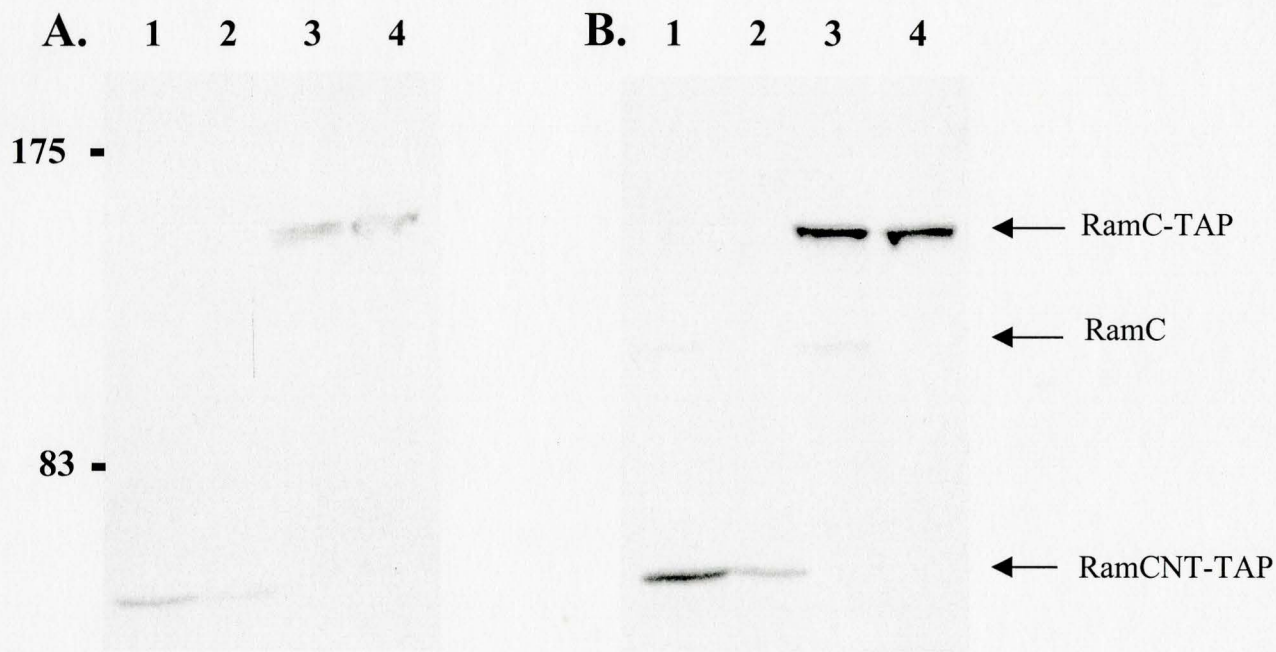


Figure 15. Expression of RamC-TAP and RamCNT-TAP in *S. coelicolor* wildtype strain M145 and *ramC* null mutant N373. The whole cell lysate of M145 + pSETΩ*ramCNT-tap* (lane1), N373 + pSETΩ*ramCNT-tap* (lane 2), M145 + pSETΩ*ramC-tap* (lane 3) and N373 + pSETΩ*ramC-tap* (lane 4), equivalent to 2mg cells, were analyzed on 8% SDS-PAGE and electroblotted onto PVDF membrane. In panel A, the PVDF membrane was probed with HRP-coupled anti-rabbit antibody only. In panel B, the PVDF membrane was probed with affinity purified anti-RamC antibody before HRP-coupled anti-rabbit secondary antibody.

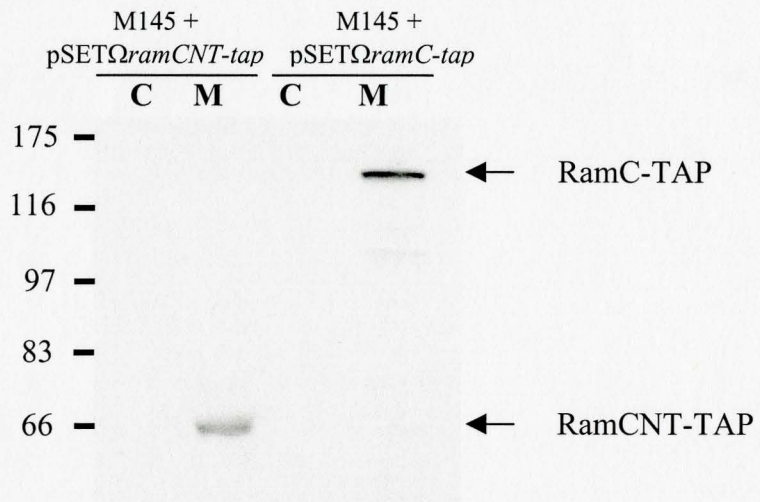
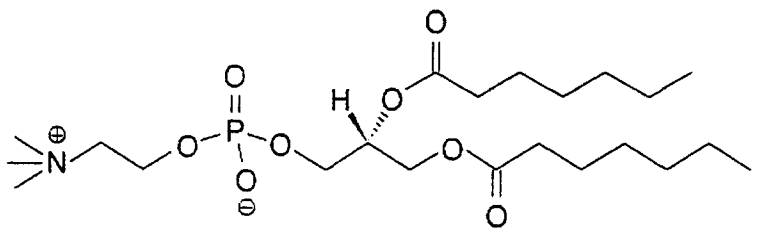
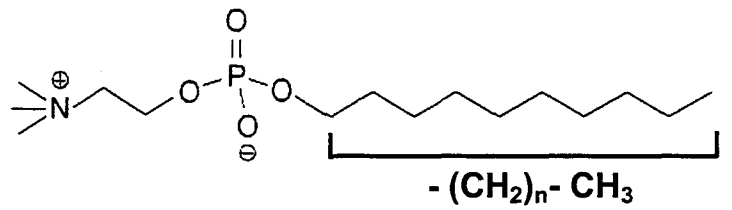


Figure 16. Subcellular localization of RamC-TAP and RamCNT-TAP in M145. Western analysis shows cytoplasmic fractions (lanes C) and membrane fractions (lanes M) of M145 strains containing pSETΩ*ramCNT-tap* and pSETΩ*ramC-tap*.

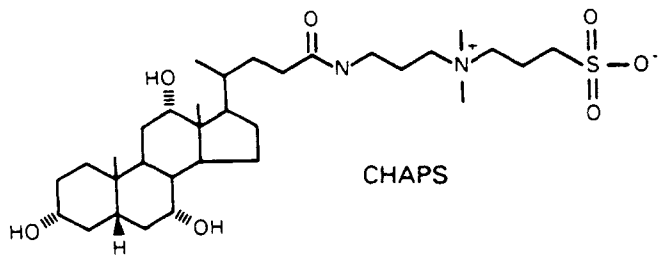
DHPC



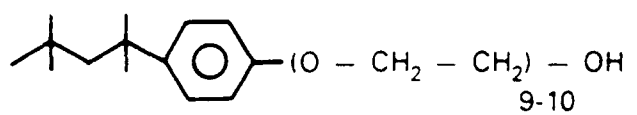
Fos - choline compound



CHAPS



Triton X-100



Triton X-100

Figure 17. Chemical form of DHPC, Fos-choline series compounds, Trition X-100 and CHAPS.

Table 6. Summary of the detergent mediated solubilization of membrane protein RamC-TAP.

Detergent	cmc (mM)	Whole Protein Concentration (mg/ml)	Detergent Concentration Range Tested	Best Detergent Concentration (mM)	Best Recovery
DHPC	1.4	1 – 5	5 – 30m M	10mM	<5%
Fos-choline 10	11	2.5	10 – 100 mM	100mM	~ 40%
Fos-choline 12	1.5	2.5	2.5 – 20 mM	10mM	~ 50%
Fos-choline 13	/	2.5	1 – 50 m M	1mM	~ 5%
Fos-choline 14	0.12	2.5	0.5 – 10 m M	0.5mM	<5%
Fos-choline 15	/	2.5	1 – 50 m M	1mM	~ 5%
Fos-choline 16	0.013	2.5	0.1 – 5 m M	0.25mM	<5%
Triton X-100	0.2	2.5	0.01% - 5%	0.1%	~ 10%
CHAPS	8	2.5	4 – 10 mM	10mM	~ 10%

cmc. Critical micelle concentration

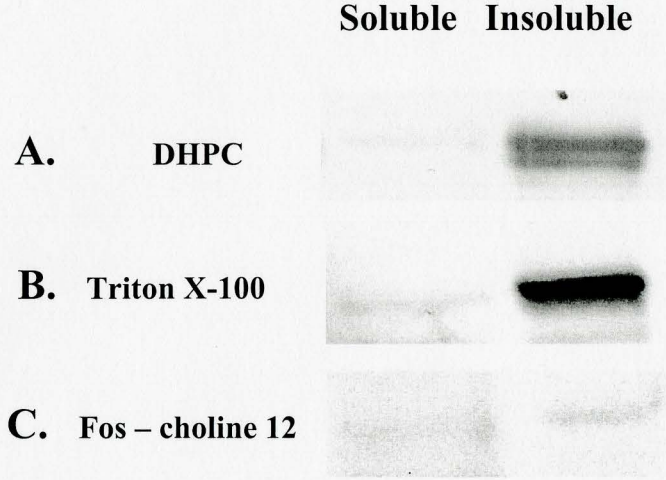


Figure 18. Detergent – mediated solubilization of RamC-TAP from membrane fraction. Western analysis indicate the best recovery of soluble RamC-TAP from membrane fraction by using DHPC (A), Triton X-100 (B) and Fos-choline 12 (C).

5.2.3 Covalent cross-linking of RamC with its binding partners

Crosslinking reagents can be interesting tools to study the interacting proteins of RamC since they are able to induce intermolecular crosslinking between interacting molecules. Dimethyl suberimidate (DMS) was used in this work. DMS is a homodiester, it possess two identical groups, which can react with primary amine groups to form stable covalent bonds.

The concentration range of DMS 10-40mM were used to crosslink RamC in *S. coelicolor* wildtype M145 and in RamC expressing *E. coli* strain BL21-(DE3)-plysS+pEMH-1. In parallel, the same crosslinking experiments were conducted in corresponding control strains that were absent in RamC production, including *S. coelicolor ramC* null mutant N373 and *E. coli* strain BL21- (DE3)-plysS containing the background plasmid pET21 (BL21- (DE3)-plysS+pET21a). The whole cell lysate of each crosslinked sample was prepared and subjected to Western analysis by using affinity purified anti-RamC antibody.

As shown in the figure 19A, with or without DMS, no RamC-specific signals appeared in N373. In M145, however, two bands (band 1 and band 2) higher than RamC were detected in the lanes with DMS treatment but absent in the lane without DMS, indicating crosslinked products. While band 2 appeared with a DMS concentration from 10mM to 40mM, band 1 could only be visualized with a DMS concentration of 20mM and 40mM. In *E. coli* strains expressing control vector BL21 (BL21- (DE3)-plysS+pET21a), no band could be detected. In contrast, in RamC expressing *E. coli* strain

BL21- (DE3)-plysS+pEMH-1, with 20mM DMS treatment, 2 bands other than RamC appeared. The two bands were labeled as band A and band B (Figure 19B).

The cell background in *S. coelicolor* and *E. coli* are totally different. If any crosslink from M145 was of same size of that from *E. coli*, it possibly revealed the potential oligomeric state of RamC. Therefore, I crosslinked RamC in M145 and *E. coli* at exactly the same time (Figure 19C) and the crosslinked products were analyzed on the same gel. However, since the RamC in the membrane fraction of *E. coli* is highly concentrated, in order to balance the RamC level, the total protein content in all of the *E. coli* lanes was much lower than those of M145. That is the reason that the RamC protein band in *E. coli* was a little bit higher than that in the M145. In the strain BL21- (DE3)-plysS + pEMH-1, there were 3 crosslinks (band A, B, C) appeared after 20mM and 40mM DMS incubation, but absent in the lane with no DMS treatment. In M145, there were two crosslinks (band 1 and band 2) detected just as seen before (Figure 19A). Band 1 seemed to migrate very closely to band A, which is bigger than 175 kDa. The molecular weight of RamC was 97 kDa, thereby band 1 or A probably resulted from a dimer of RamC or even higher oligomeric state. However, it is not accurate to estimate the molecular weight of band 1/A due to the migration of such a large protein complex on the 8% SDS-PAGE usually does not relate very well to its molecular weight. Some other experiments need to be done to provide more evidences for the hypothesis. Possible techniques include gel filtration, analytical ultracentrifugation, etc. (Eccleston *et al.*, 2002, Shier *et al.*, 2001).

Another objective of the chemical cross-linking is to identify interacting proteins of RamC. We surmised that band 2 which appeared in M145 but absent in RamC expressing *E. coli* strain revealed one or more interacting partners of RamC.

Mike Hudson has prepared several carboxyl terminal deletion of *ramC* (unpublished data). Once those truncated *ramC* are expressed in *S. coelicolor*, the chemical crosslinking experiment can be performed in those strains to reveal the binding domain of RamC with its partner or itself.

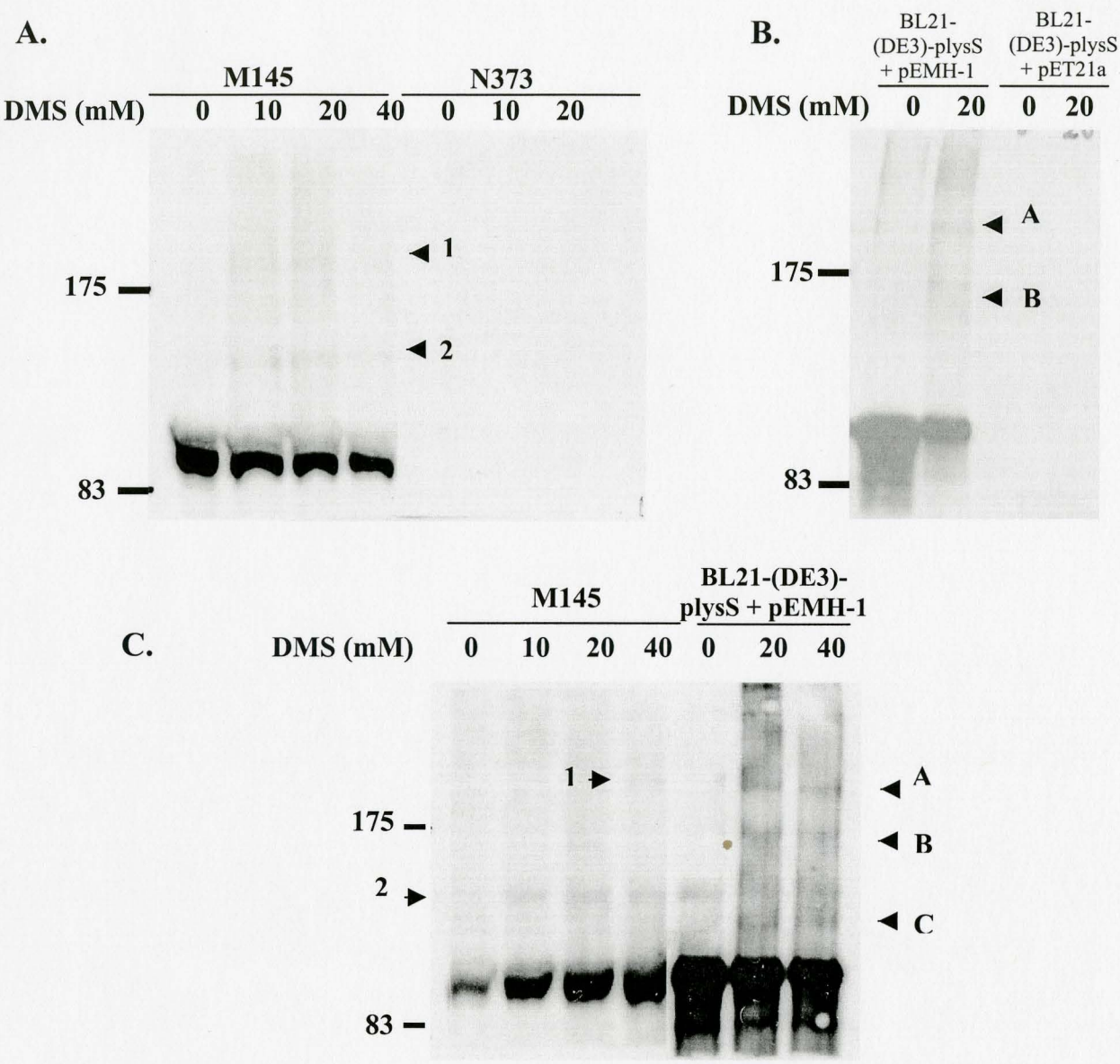


Figure 19. Identification of RamC crosslinking products in M145 and RamC expressing *E. coli* strain (BL21- (DE3)-plysS + pEMH-1) by using DMS. A). Western analysis show the RamC crosslinks in the cell pellet of *Streptomyces coelicolor* wildtype M145 and *ramC* null mutant N373. B). Western analysis shows the RamC crosslinks in the membrane fraction of RamC expressing *E. coli* strain (BL21- (DE3)-plysS + pEMH-1) and *E. coli* strain containing the background vector (BL21- (DE3)-plysS + pET21a). C). Comparison of the RamC crosslinks in M145 and BL21- (DE3)-plysS + pEMH-1.

6.0 References

Ainsa, J.A., Parry, H.D., Chater, K.F. (1999). A response regulator-like protein that functions at an intermediates stage of sporulation in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* **34**: 607-19

Ainsa, J.A., Ryding, N.J., Hartley, N., Findlay, K.C., Bruton, C.J. and Chater K.F. (2000). WhiA, a protein of unknown function conserved among gram-positive bacteria, is essential for sporulation in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **182**: 5470-8

Bakal, C.J. and Davies, J.E. (2000). No longer an exclusive club: eukaryotic signaling domains in bacteria. *Trends Cell Biol.* **10**: 397-408

Bentley, S.C. *et al.*, (2002). Complete genome sequence of the model actinomycetes *Streptomyces coelicolor* A3(2). *Nature* **417:** 141-7.

Bibb, M. J., Molle, V. and Buttner, M. (2000). σ^{BldN}, an extracytoplasmic function RNA polymerase sigma factor required for aerial mycelium formation in *Streptomyces coelicolor* A(3)2. *J. Bacteriol.* **182:** 4606 –16.

Bignell, D. R., Warawam J. L., Strap, J. L. Chater, K. F. and Leskiw, B. K. (2000). Study of the *bldG* locus suggests that an anti-anti-sigma factor and an anti-sigma factor may be involved in *Streptomyces coelicolor* antibiotic production and sporulation. *Microbiology* **146:** 2161-73.

Champness, W.C. (1988). New loci required for *Streptomyces coelicolor* morphological and physiological differentiation. *J. Bacteriol.* **170**(3): 1168-74

Chater, K. F. (1972). A morphological and genetic mapping study of white colony mutants of *Streptomyces coelicolor. J. Gen. Microbiol.* **72**: 9-28

Chater, K. F. (1975). Construction and phenotypes of double sporulation deficient mutants in *Streptomyces coelicolor* A3(2). *J.Gen. Microbiol.* **87**: 312-25

Chater, K. F. (1989). The developmental fate of *S. coelicolor* hyphae depends upon a gene product homologous with the motility sigma factor of *B. subtilis*. *Cell* **59**:133-43

Chater, K. F. (1993). Genetic of differentiation in *Streptomyces*. *Annu. Rev. Microbiol.* **47**:685-713

Chater, K.F. (1998). Taking a genetic scalpel to the *Streptomyces* colony. *Microbiology*, **144**: 1465 – 1478

Criekinge V.W. and Beyaert R. (1999). Yeast Two-Hybrid: State of the Art. *Biol. Proc. Online* **2**:1-38

Davis N.K. and Chater K.F. (1990). Spore colour in *Streptomyces coelicolor* A3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. *Mol. Microbiol.* **4**:1679-91

de Crecy-Lagard V, Servant-Moisson P, Viala J, Grandvalet C, Mazodier P. (1999). Alteration of the synthesis of the Clp ATP-dependent protease affects morphological and physiological differentiation in *Streptomyces*. *Mol Microbiol.* **32**:505-17.

Eccleston, M., Ahmed Ali, E., Seyler, R., Westpheling, J., and Nodwell, J. (2002). Structural and Genetic analysis of the BldB protein of *Streptomyces coelicolor. J. Bacteriology,* **184**: 4270-6

Elliot, M. A. and B. K Leskiw. (2001). BldD is a direct regulator of key developmental genes in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* **40(1):** 257-69

Flardh, K., Findlay, K.C., and Chater K.F. (1999). Association of early sporulation genes with suggested developmental decision points in *Streptomyces coelicolor* A3(2). *Microbiology*. **145**: 2229-43.

Hanks, S., Quinn, A.M. and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **24**: 42-52

Hopwood, D.A., Wildermuth, H. and Palmer, H.M. (1970). Mutants of *Streptomyces coelicolor* defective in sporulation. *J. Gen. Microbiol.* **61**: 397-408

Horinouchi S. and Beppu T., (1994). A-factor as a microbial hormone that controls cellular differentiation and secondary metabolism in *Streptomyces griseus. Mol. Microbiol.* **12**: 859-864

Hudson, M.E., Zhang, D., and Nodwell, J.R., (2002). Membrane association and kinase-Like motif of the RamC protein of *Streptomyces coelicolor. J. Bacteriol.* **184**: 4920 – 4

Karimova, G., Pidoux, J., Ullmann, A., and Ladant D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Microbiology* **95:** 5752 –6

Kelemen, G.H. and Buttner, M.J. (1998). Initiation of aerial mycelium formation in *Streptomyces. Curr. Opin. Microbiol.* **1**:656-62

Kelemen, G.H., Brown, G.L., Kormanec, J., Potuckova, L., Chater, K.F., and Buttner, M.J. (1996). The positions of the sigma-factor genes, whiG and sigF, in the hierarchy controlling the development of spore chains in the aerial hyphae of *Streptomyces coelicolor* A3(2*). Mol. Microbiol.* **21**:593-603

Keijser, B.J., Wezel, G.P. and Vijgenboom, E. (2002). Developmental regulation of the *Streptomyces lividans ram* genes: involvement of RamR in regulation of the *ramCSAB* operon. *J. Bacteriology* **184**: 4420 - 9.

Kessi, J., Poiree, C., Wehrli, E., Rachofen, R., Semenza, G.M. and Hauser, H., (1994) Short-chain phosphatidylcholines as superior detergents in solubilizing membrane proteins and preserving biological activity. *Biochemistry* **33:** 10825-36

Khokhlov, A.S., Tovarova, I.I., Borisova, L.N., Pliner, S.A., Shevchenko, L.N., Kornitskaia, E.I., Ivkina, N.S. and Rapoport, I.A. (1967) The A-factor, responsible for streptomycin biosynthesis by mutant strains of actinomyces streptomycin. *Dokl Akad Nauk SSSR.* **177**(1):232-5.

Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. and Hopwood, D. A. (2000). *Practical Streptomyces Genetics*. Norwich, England: John Innes Foundation.

Ma, H. and Kendall K. (1994). Cloning and analysis of a gene cluster from *Streptomyces coelicolor* that causes accelerated aerial mycelium formation in *Streptomyces lividans*. *J Bacteriol.* **176**(12):3800-11.

MacNeil, D.J., Gewain, K.M., Ruby, C.L., Dezeny, G., Gibbons, P.H., and MacNeil, T. (1992). Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **11**: 61 – 8.

Matsumoto, A., Hong, S.K., Ishizuka, H., Horinouchi, S., and Beppu, T. (1994). Phosphorylation of the AfsR protein involved in secondary metabolism in *Streptomyces* species by a eukaryotic-type protein kinase. *Gene.* **146**(1):47-56.

Mendez, C. & Chater, K.F. (1987). Cloning of *whiG*, a gene critical for sporulation of *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **169**: 5715-5720

Merrick M.J. (1976). A morphological and genetic mapping study of bald colony mutants of *Streptomyces coelicolor. J. Gen. Microbiol.* **96**: 299-315

Miguelez E.M., Hardisson, C. and Manzanal, M. B. (1999). Hyphal Death during colony development in *Streptomyces* antibiotics: morphological evidence for the existence of a process of cell deletion equivalent to apoptosis in a multicellular prokaryote. *J. Cell. Biol.* **145:** 515-525.

Miguelez, E. M., Hardisson, C. and Manzanal, M. B. (2000). *Streptomyces:* a new model to study cell death. *Int. Microbiol.* **3:** 153-158.

Miyake K, Horinouchi S, Yoshida M, Chiba N, Mori K, Nogawa N, Morikawa N, Beppu T. (1989) Detection and properties of A-factor-binding protein from *Streptomyces griseus. J. Bacteriol.* **171**(8):4298-302.

Molle, V., and Buttner, M. J. (2000). Different alleles of the response regulator gene *bldM* arrest *Streptomyces coelicolor* development at distinct stages. *Mol. Microbiol.* **36:** 1265 –78.

Ninfa, E.G., Atkinson, M.R., Kamberov, E.S. and Ninfa A.J. (1993). Mechanism of autophosphorylation of *Escherichia coli* nitrogen regulator II (NR_{II} or NtrB): *trans*-phosphorylation between subunits. *J. Bacteriol.* **175**: 7024 - 32

Nodwell, J., Kuo, D., Yang, M., and Losick, R. (1999). Extracellular complementation and the identification of additional genes involved in aerial mycelium formation in *Streptomyces coelicolor. Genetics,* **151**: 569-84

Nodwell, J. and Losick, R. (1998). Purification of an extracellular signaling molecule involved in production of aerial mycelium by *Streptomyces coelicolor*. *J. Bacteriol.*, **180**, 1334-7

Nodwell, J., McGover, K. and Losick, R. (1996). An oligopeptide permease responsible for the import of an extracellular signal governing aerial mycelium formation in *Streptomyces coelicolor*. *Mol. Microbiol.*, **22**(5): 881-93.

O'Connor, I., Kannellis, P., and Nodwell, J.R., (2002) The *ram*C gene is required for morphogenesis in *Streptomyces coelicolor* and expressed in a cell type-specific manner under the direct control of RamR *Mol. Microbiol.* **45:** 45 – 57

Onaka, H., Nakagawa, T., and Horinouchi, S. (1998). Involvement of two A-factor receptor homologues in *Streptomyces coelicolor* A(3)2 in the regulation of secondary metabolism and morphogenesis. *Mol. Microbiol.* **28:** 743-53.

Onaka, H., Ando, N., Nihira, T., Yamada Y., Beppu, T. and Horinouchi, S. (1995). Cloning and characterization of the A-factor receptor gene from *Streptomyces griseus*. *J. Bacteriol.* **177**: 6083-92

Pan, S.Q., Charles, T., Jin, S., Wu, Z.L. and Nester E.W. (1993). Performed dimeretic state of the sensor protein VirA is involved in plant-*Agrobacterium* signal transduction. *Proc, Natl. Acad. Sci. USA* **90**: 9903-43

Pope, M.K., Green, B.D., and Westpheling J. (1998) The *bldB* gene encodes a small protein required for morphogenesis, antibiotic production, and catabolite control in *Streptomyces coelicolor*. *J. Bacteriol.* **180**: 1556-62

Potter, M.D. and Nicchitta, C.V. (2000) Ribosome-independent regulation of translocon composition and Sec61α conformation. *J. Biol. Chem.* **275:** 2037 – 45

Potúcková, L., Kelemen, G.H., Findlay, K.C., Lonetto, M.A., Buttner, M.J. And Kormanec, J. (1995). A new RNA polymerase sigma factor, sigma F, is required for the late stages of morphological differentiation in *Streptomyces spp. Mol. Microbiol.* **17**(1): 37-48

Puig, O., Caspary, F., Rigut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and Séraphin, B., (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* **24**: 218 – 29

Rigaut, G., Schevchenko, A., Rutz, B., Wilm, M., Mann, M., and Séraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol* **17:** 1030-2

Ryding NJ, Kelemen GH, Whatling CA, Flardh K, Buttner MJ, Chater KF. (1998) A developmentally regulated gene encoding a repressor-like protein is essential for sporulation in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* **29**(1):343-57.

Schivanna, B.D., and Rowe, E.S. (1997) Preservation of the native structure and function of Ca^{2+}-ATPase from Sarcoplasmic tericulum: solubilization and reconstitution by new short-chain phospholipid detergent 1,2-diheptanoyl-*sn*-phospharidylcholine. *Biochem. J.* **325:** 533–42

Shier, V.K., Hancey, C.J., Benkovic, S.J., (2001) Identification of the active oligomeric state of an essential adenine DNA methyltransferase from *caulobacter crescentus. J. Biol. Chem.* **276**(18): 14744 – 51

Skamnaki V.T., Owen D.J., Noble M.E., Lowe E.D., Lowe G, Oikonomakos N.G., Johnson L.N. (1999). Catalytic mechanism of phosphorylase kinase probed by mutational studies. *Biochemistry.* **38**(44):14718-30

Swanson, R.V., Bourret, R.B. and Simon, M.I. (1993). Intermolecular complementation of the kinase activity of CheA. *Mol. Microbiol.* **8**: 435- 41

Tillotson R.D., Wosten H.A., Richter M, Willey J.M. (1998). A surface active protein involved in aerial hyphae formation in the filamentous fungus Schizophillum commune restores the capacity of a bald mutant of the filamentous bacterium *Streptomyces coelicolor* to erect aerial structures. *Mol Microbiol.* **30**(3):595-602

Ueda K., Umeyama T., Beppu T. and Horinouchi S. (1996). The aerial mycelium-defective phenotype of *Streptomyces griseus* resulting from A-factor deficiency is suppressed by a Ser/Thr kinase of *S. coelicolor* A3(2). *Gene.* **169**: 91-5

Willey J.W., Santamaria R., Guijarro J., Geislich M. and Losick R. (1991). Extracellular complementation of a developmental mutation implicates a small sporulation protein in aerial mycelium formation by *S. coelicolor. Cell.* **65**: 641-50

Willey, J. W., Schwedock, J., and Losick, R. Losick (1993). Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation in *Streptomyces coelicolor. Genes Dev* **7:** 895-903.

Wolfe, A.J. and Stewart, R.C. (1993) The short form of the CheA protein restores kinase activity and chemotactic ability to kinase-deficient mutants. *Proc. Natl. Acad. Sci. USA* **90**: 1518-22

Yang, Y. and Inouye, M. (1992) Intermolecular complementation between two defective mutant signal-transducing receptors. *Proc. Natl. Acad. Sci. USA* **88**: 11057-61

Yu, D., Ellis, H. M., Lee, E., Jenkins, N. A., Copeland N. G., and Court D. L. (2000) An efficient recombination system for chromosome engineering in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **97**, 5978 – 83

Yu, T.W. & Hopwood, D.A (1995). Ectopic expression of the *Streptomyces coelicolor whiE* genes for polyketide spore pigment synthesis and their interaction with the *act* genes for actinorhodin biosynthesis. *Microbiology* **141:** 2779 – 91

Zheng, J, Knighton, D.R., ten Eyck, L.F., Karlsson, R., Xuong, N., Taylor, S.S., Sowadski, J.M. (1993). Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. *Biochemistry* 1993, **32:** 2154-61