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# Osmotic and growth-phase dependent regulation of the *eta* gene of *Staphylococcus aureus*: a role for DNA supercoiling

**Brian J. Sheehan [1], Timothy J. Foster [1], Charles J. Dorman [2], Simon Park [3], and Gordon S.A.B. Stewart [4]**

[1] Department of Microbiology, Moyne Institute, Trinity College, Dublin 2, Republic of Ireland
[2] Molecular Genetics Laboratory, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, UK
[3] Department of Microbiology, A.F.R.C. Institute of Food Research, Reading Laboratory, Shinfield, Reading RG2, 9AT, UK
[4] Department of Applied Biochemistry and Food Science, School of Agriculture, University of Nottingham, Sutton Bonington, Loughborough, LE12 5RD, UK

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**Summary.** Transcriptional fusions were constructed between the promoter for the epidermolytic toxin A (*eta*) gene of *Staphylococcus aureus* and the *luxAB* and *xylE* reporter gene systems. The expression of the fusion products was found to be dependent upon the accessory gene regulator (*agr*) locus and was observed to increase significantly during the transition from the exponential to the stationary phase of growth. Furthermore the expression of the *eta* gene promoter was found to be osmotically regulated, with the expression levels of the *eta* fusions being inversely related to the osmolyte levels. The ability of environmental factors to influence DNA topology (and thence gene expression) was investigated. High osmolarity (0.7 M NaCl) resulted in an increase in the degree of negative supercoiling of plasmid DNA in the *S. aureus* strain 8325-4 ($Agr^+$) but not in strain ISP546 ($Agr^-$). Furthermore the *eta* promoter was strongly induced in *S. aureus* cultures grown in the presence of sub-inhibitory concentrations of novobiocin, a DNA gyrase inhibitor. However this induction was independent of *agr*, suggesting that the *eta* promoter is subject to both *agr*-dependent (osmolarity, growth phase) and -independent (DNA topology) regulatory processes.

**Key words:** *Staphylococcus aureus* – Gene regulation – Epidermolytic toxin A – Osmoregulation – DNA supercoiling

## Introduction

*Staphylococcus aureus* produces a variety of extracellular and cell-bound proteins, which have been implicated in the pathogenesis of staphylococcal disease (for reviews see Easmon and Adlam 1983). Mutations in the accessory gene regulator (*agr*) locus result in a pleiotropic exoprotein-deficient phenotype (Morfeldt et al. 1988; Peng et al. 1988). One such mutation is a transposon Tn*551* insertion in *agr*, which results in a decrease in the post-exponential phase production of $\alpha,\beta$ and $\delta$-haemolysins, TSST-1 (toxic shock syndrome toxin 1), the epidermolytic toxins ETA and ETB and enterotoxins B and D, while the production of coagulase and protein A is increased (Bjorklind and Arvidson 1980; Rescei et al. 1986; O'Toole and Foster 1986; Gaskill and Khan 1988; Bayles and Iandolo 1989; Mahmood and Khan 1990). The *agr* locus is believed to act primarily at the level of transcription and this effect is exerted *in trans* (Janzon et al. 1986; Rescei et al. 1986).

Sequence analysis of the cloned *agr* locus has revealed that four open reading frames, *agrA, agrB, agrC* and *agrD* are transcribed divergently from *hld*, the gene encoding $\delta$-haemolysin (Janzon et al. 1989). The *agrA* gene containing the site of the Tn*551* insertion is transcribed from a weak constitutive promoter (P1), and is also expressed as part of a polycistronic transcript (including *agrBCD*) initiated 2 kb upstream at promoter P2. Both P2 and the *hld* promoter P3 are strongly growth-phase regulated. The *agrA* gene encodes a protein that has sequence homology with the response regulator proteins of two-component signal transduction systems. Furthermore, the *agrB* product is homologous to the histidine protein kinase partner (Stock et al. 1989). It is proposed that the regulatory protein, AgrA, stimulates transcription from P2 and P3, possibly by interaction with an inverted repeat situated midway between P2 and P3 (Janzon et al. 1989). The role of the $\delta$-haemolysin mRNA as a major regulator of exoprotein synthesis has been demonstrated (Janzon and Arvidson 1990), but little is known about the mechanisms involved.

The role of two-component signal transduction systems in the adaptive responses of prokaryotes to environmental stress is becoming increasingly evident (for reviews see Stock et al. 1989, 1990; Miller et al. 1989; Ronson et al. 1987). We report here the effect of osmotic stress on the *agr*-regulated expression of the promoter for the epidermolytic toxin A gene (*eta*). The gene encoding ETA has been cloned and sequenced (O'Toole and Foster 1987; Lee et al. 1987; Sakuri et al. 1988). The

*Offprint requests to:* T.J. Foster

expression of the cloned toxin gene has been shown to be dependent on the integrity of the *agr* locus, with production of the toxin increasing significantly in stationary phase cultures (O'Toole and Foster 1987). Here we describe the fusion of the *eta* promoter region to two different reporter genes, the luciferase (*luxAB*) genes of *Vibrio fischeri* and the catechol 2,3-dioxygenase (*xylE*) gene from *Pseudomonas putida*. The effect of varying osmolyte concentrations on the expression of both the *eta-luxAB* and *eta-xylE* fusions has been investigated in $Agr^+$ and $Agr^-$ host strains. Furthermore the role of DNA supercoiling in the *agr*-regulated expression of *eta* has been studied.

## Materials and methods

*Bacterial strains and plasmids.* The bacterial strains used are listed in Table 1 and the plasmids are given in Table 2.

*Bacterial growth media. Escherichia coli* strains were grown in L broth and agar (Maniatis et al. 1982). *S. aureus* strains were routinely cultured in trypticase soy broth (TSB) and agar (TSA, Oxoid) incorporating chloramphenicol to 10 μg/ml. The salt concentration of commercial TSB is 0.086 M. In order to vary the osmolarity of the growth medium, 2× concentrated TSB lacking NaCl was prepared with tryptone (Difco) 34 g, neutralised soya peptone (Oxoid) 6 g, D-glucose (BDH) 5 g and $K_2HPO_4$ (BDH) 5 g per litre. Subsequently normal strength TSB was prepared with concentrations of NaCl ranging from 0 M to 1.0 M. Alternatively, sucrose was added to final concentrations of 0.44 M.

*DNA manipulations.* DNA modifying enzymes were purchased from either the Boehringer Corporation or Promega and were used according to the manufacturers' recommendations. DNA manipulations were performed using standard procedures (Maniatis et al. 1982).

**Table 1.** Bacterial strains

| Strain | Genotype or properties | Reference |
|---|---|---|
| *Escherichia coli* | | |
| TB1 | host for detecting chimaeric pUC plasmids | Bethesda Research Laboratories |
| JM101 | *lac-pro, thi, supE*44, *endA*, *gyrA*96, *hsd*17, *recA*1, F′ *traD*36, *proAB*, *lacI*$^q$ *lacZ*ΔM15 | (Yanisch-Perron et al. 1985) |
| *Staphylococcus aureus* | | |
| 8325-4 | $Agr^+$ NCTC8325 cured of prophages | (Novick 1963) |
| RN4220 | mutant of 8325-4 capable of accepting shuttle plasmids | (Kreiswirth et al. 1983) |
| ISP546 | *agr*::Tn*551* $Agr^-$ mutant of 8325-4 | (Mallonee et al. 1982) |

*Transfer of plasmids.* Plasmids were transformed into *E. coli* cells made competent by treatment with $CaCl_2$ (Maniatis et al. 1982) and into *S. aureus* strains by electroporation (Oskouian and Stewart 1990).

*Enzyme assays.* Luciferase activity was measured using either a Turner Designs 20 luminometer or an LKB model 1250 luminometer. Cultures were grown in 500 ml conical flasks containing 50 ml of TSB and incubated at 30° C with shaking. At regular intervals, 1 ml samples were removed and assayed for luminescence. As pSP100 does not contain the genes for production of an aldehyde necessary for luciferase activity (Meighen 1988), 10 μl of dodecyl aldehyde (Sigma Chemical Co., $C_{12}$; 1%, v/v in ethanol) was added prior to measurement.

**Table 2.** Plasmids

| Plasmid | Host | Markers[a] of antibiotic resistance | Other properties | Reference |
|---|---|---|---|---|
| pEP2 | *E. coli* | Ap | pUC19 with *Eco*RI-*Pst*I/blunt fragment carrying *eta* promoter | (Patel et al., personal communication) |
| pLC4 | *S. aureus*/*E. coli* | Cm, Ap | Promoter probe vector containing *xylE* gene | (Ray et al. 1985) |
| pXL2 | *S. aureus* | Cm, Ap | *eta-xylE* fusion plasmid | This study |
| pKSB7 | *S. aureus* | Cm | Uncharacterised *Lactobacillus casei* promoter fragment-*luxAB* fusion | (Ahmed and Stewart 1991) |
| pSB71 | *E. coli* | Ap | pBR322 based promoterless *lux* expression vector. Unique *Eco*RI promoter cloning site | (Ahmed and Stewart 1991) |
| pSB197 | *S. aureus*/*E. coli* | Cm, Ka | *E. coli*/*S. aureus* shuttle vector containing promoterless *lux* genes from pSB71 | This study |
| pSB204 | *S. aureus* | Cm, Ka | Promoter probe vector containing promoterless *luxAB* genes from *Vibrio fischeri* | This study |
| pSP100 | *S. aureus* | Cm, Ka | *eta-luxAB* fusion plasmid | This study |
| pBS1 | *S. aureus* | Cm | Derivative of pCW59 lacking tetracycline gene | This study |

[a] Ap, ampicillin; Cm, chloramphenicol; Ka, kanamycin

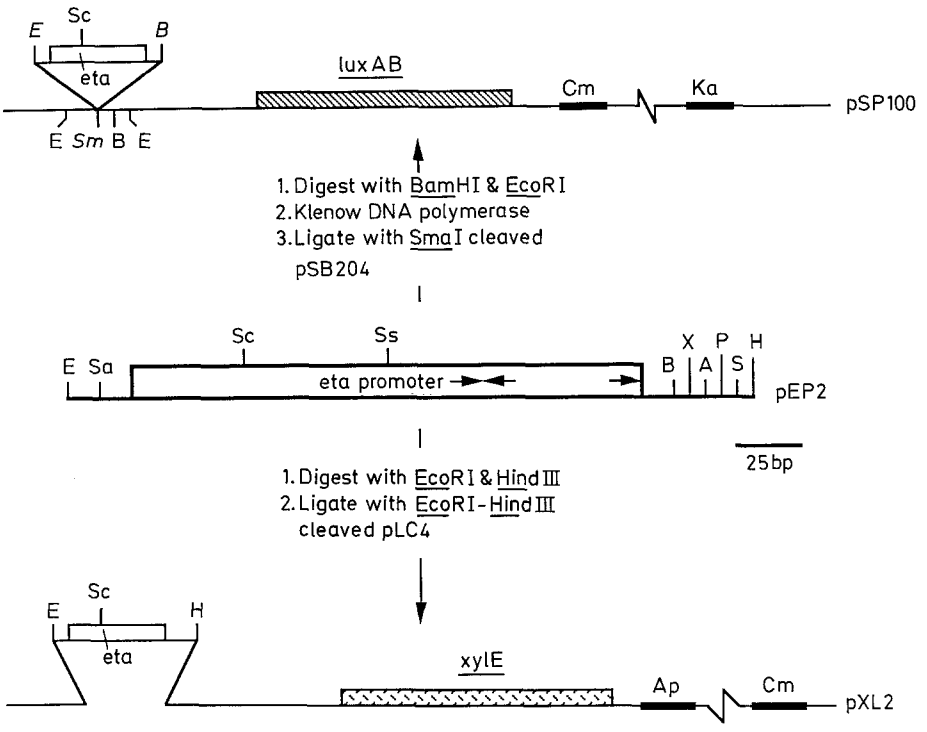



**Fig. 1.** Construction of plasmids pSP100 (*above*) and pXL2 (*below*). The *eta* promoter region used to generate the transcriptional fusions is shown to scale, flanked by the remaining restriction enzyme sites of the pUC19 multiple cloning site. The *arrows* represent inverted repeat sequences near which are located two possible −35, −10 consensus promoter sequences. Neither pSP100 nor pXL2 are drawn to scale. Restriction site abbreviations: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sph*I, Sa, *Sac*I; Sc, *Sca*I; Sm, *Sma*I; Ss, *Ssp*I; X, *Xba*I. Abbreviations in *italics* represent restriction sites that are not present in the final construct

$OD_{540\,nm}$ of the cultures was determined at the time of sampling. Catechol 2,3-dioxygenase assays were performed using a method adapted from Zukowski et al. (1983). Cultures (20 ml) were grown in 250 ml conical flasks for 18 h at 37° C with shaking. Samples of 10 ml were harvested and washed with 2 ml of 20 mM potassium phosphate buffer (pH 7.2). Cells were suspended in 2 ml lysis buffer (100 mM potassium phosphate pH 7.5, 20 mM EDTA, 10% acetone and 37 μg/ml lysostaphin) and incubated at 37° C for 20 min. Following the addition of 10% Triton X-100, the lysate was placed on ice for 15 min and then centrifuged for 20 min at 27000 *g* to pellet the cellular debris. Catechol 2,3-dioxygenase activities were determined spectrophotometrically at $375_{nm}$ and 37° C, in a total volume of 3 ml which contained 0.25 ml of cell extract and 2.75 ml of 100 mM potassium phosphate (pH 7.5) incorporating catechol to 0.2 mM. Specific activities were calculated where one milliunit corresponds to the formation of 1 millimole of 2-hydroxymuconic semialdehyde per minute at 37° C.

*Construction of* eta-luxAB. The *eta* promoter region had previously been localised to a 220 bp sequence (O'Toole and Foster 1987) and cloned into pUC19 between the *Sac*I and *Sma*I sites in the multiple cloning site to create the plasmid pEP2 (Patel et al., manuscript in preparation). A *lux* promoter probe vector for *S. aureus* was constructed as follows: pCK1 (Gasson and Anderson 1985) was digested with *Eco*RI and *Pvu*II and the resultant 4.3 kb fragment containing both the origin of replication and antibiotic resistance markers was isolated. A 2158 bp *luxAB Eco*RI-*Sma*I cassette was isolated from pSB71 (Ahmed and Stewart 1988) and ligated to the 4.3 kb fragment from pCK1. The resultant recombinant was designated pSB197. The oligonucleotide 5′-AATTCCCGGGGATCCG-3′ was subsequently inserted into the unique *Eco*RI site of pSB197 to give pSB204.

To generate the *eta-luxAB* fusion the promoter region was removed from pEP2 by cleaving with *Eco*RI and *Bam*HI (Fig. 1). DNA polymerase I (Klenow fragment) was used to create blunt ends and the promoter-bearing fragment was cloned into the unique *Sma*I site of pSB204, forming pSP100. Following transformation of *E. coli* JM101 cells, bioluminescent colonies were identified using a Hamamatsu Argus 100 Vim 3 photon video camera. Plasmid DNA purified from *E. coli* was subsequently used to transform *S. aureus* strains, with transformants selected on TSB agar containing 10 μg/ml chloramphenicol.

*Construction of* eta-xylE. The *eta-xylE* fusion plasmid was derived from the expression vector pLC4 (Ray et al. 1985). While the *xylE* gene of pLC4 is expressed in *E. coli*, production of catechol 2,3-dioxygenase in *S. aureus* is dependent on the introduction, immediately 5′ to *xylE*, of a promoter that is functional in the gram-positive host. A 260 bp *Eco*RI-*Hin*dIII fragment carrying the *eta* promoter from pEP2 was ligated with pLC4 cleaved with the same enzymes. The DNA was transformed into *S. aureus* RN4220 by electroporation. Colonies expressing catechol 2,3-dioxygenase were identified by spraying plates with an aqueous solution of 0.5-M catechol. One plasmid, pXL2, was chosen for further study.

*Construction of pBS1, a reporter plasmid for topoisomer studies.* Plasmid pCW59 (Wilson et al. 1981) was cleaved with *Hin*dIII and a 3 kb fragment carrying the chloramphenicol resistance gene and the replication functions was purified following agarose gel electrophoresis. After

ligation the DNA was transformed into *S. aureus* RN4220 by electroporation selecting for chloramphenicol resistance.

*Separation of topoisomers.* Plasmid topoisomers were isolated by electrophoresis for 50 volt hours in 1.0% agarose gels which contained chloroquine to 25 μg/ml. Following electrophoresis, the chloroquine was removed by rinsing for 4 h in water. The DNA was subsequently visualised by staining with ethidium bromide (5 μg/ml).

## Results

### agr *regulation of* eta *fusions*

In order to study environmental and other factors that influence the Agr-dependent expression of the *eta* gene, transcriptional fusions were constructed between the *eta* promoter and two reporter gene systems. Fusions with both the luciferase (*luxAB*) and catechol 2,3-dioxygenase (*xylE*) reporters were required to monitor gene expression most effectively. The *eta-luxAB* fusion was used to measure real-time changes in promoter activity during the exponential and early stationary phases of growth. However in stationary phase, the bioluminescent phenotype becomes dependent on the availability of $FMNH_2$ rather than promoter activity (particularly in broth cultures). Consequently the second fusion plasmid, pXL2, was constructed and used to monitor gene expression in stationary phase cells.

The fusion plasmids pXL2 (*eta-xylE*) and pSP100 (*eta-luxAB*) were transformed into the *S. aureus* strains 8325-4 ($Agr^+$) and ISP546 ($Agr^-$) by electroporation. The expression of luciferase by pSP100 was 100-fold higher in the $Agr^+$ host 8325-4 than in the $Agr^-$ host ISP546 (Fig. 2). Similarly the expression of catechol 2,3-dioxygenase by pXL2 was 6-fold higher in the $Agr^+$ host compared to the $Agr^-$ host strain (data not shown). This demonstrates that transcription from the *eta* promoter is positively regulated by *agr*. The growth phase dependence of *eta* expression is also apparent in Fig. 2, with the rate of transcription of the *eta-luxAB* fusion increasing significantly during the transition from the exponential phase of growth to stationary phase.

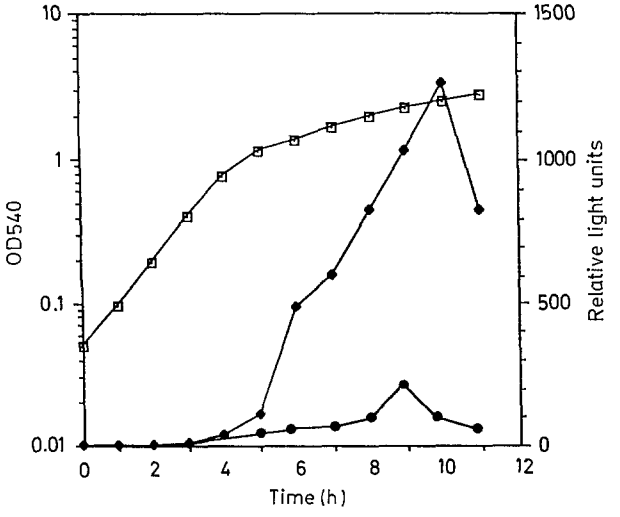
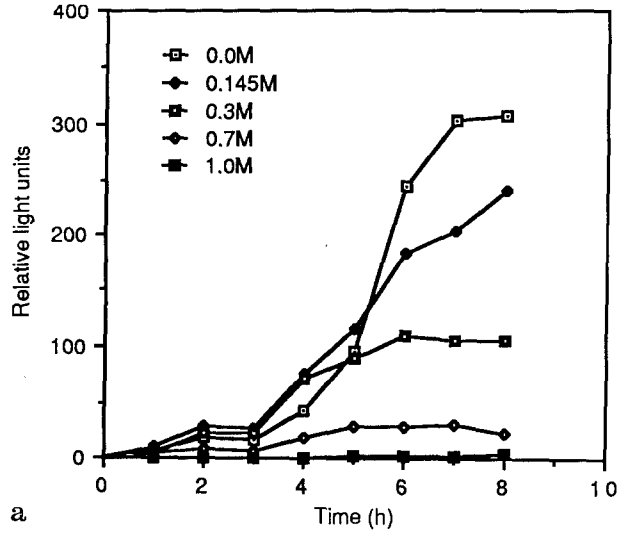


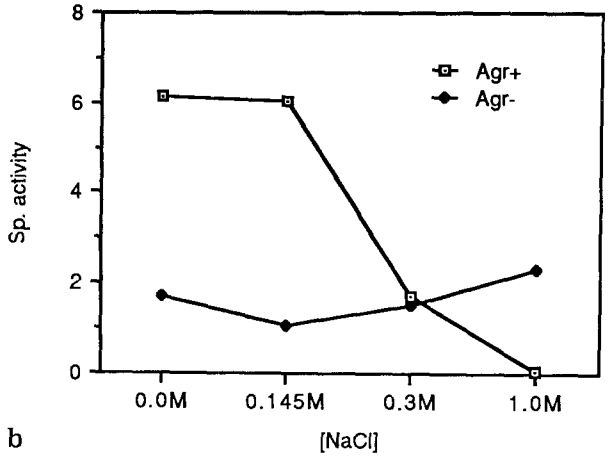

**Fig. 2.** Agr regulation of *eta-luxAB* expression. *Staphylococcus aureus* strains 8325-4 ($Agr^+$) and ISP546 ($Agr^-$) harbouring the plasmid pSP100 were grown in trypticase soy broth (TSB) and assays performed as described in the Materials and methods. (■), 8325-4 ($Agr^+$); (●), ISP546 ($Agr^-$); (□) growth as measured by optical density at 540 nm

### *Osmoregulation of* eta *expression*

The effect of varying the osmolarity of the growth medium on the expression of the *eta* fusions was investigated. In this study the *eta-luxAB* fusion was used to monitor gene expression during exponential and early stationary phase growth (Fig. 3a), while the *eta-xylE* fusion was used to monitor expression in stationary phase cultures (Fig. 3b). The highest level of expression was observed with cultures grown in TSB without NaCl. As the concentration of NaCl was increased from 0 M to 1 M, the level of expression of the *eta* fusions decreased dramatically in the $Agr^+$ host (Fig. 3a and b). Varying the osmolyte concentration had little effect on the low level of expression in the $Agr^-$ host (Fig. 3b). Interestingly at high NaCl concentrations (1.0 M), the level of expression of the *eta-xylE* fusion in the $Agr^+$ strain 8325-4 was less than that observed in the $Agr^-$ strain grown under similar conditions (Fig. 3b). As a control similar experiments were performed with $Agr^+$ and $Agr^-$ strains containing the plasmid pKSB7 (Ahmed and Stewart 1991). Under normal growth conditions this fusion is not dependent on the Agr locus for maximal expression. Furthermore, increasing the osmolarity of the growth medium from 0 M to 0.7 M NaCl, resulted in an increase in the levels of expression of this fusion construct in both $Agr^+$ and $Agr^-$ cells (Fig. 3c).

In order to determine if the expression of *eta* was dependent on osmolarity rather than $Na^+$ or $Cl^-$ ion stress, similar experiments were performed using sucrose as the osmolyte. In the $Agr^+$ strain 8325-4, the *eta-luxAB* fusion was expressed to similar levels when grown in TSB containing 0.3 M NaCl or 0.44 M sucrose for up to 8 h (Fig. 4a, upper panel). However when luciferase production was assayed on TSA plates (containing 0.3 M NaCl or 0.44 M sucrose) after 18 h growth, sucrose was observed to repress the expression of the *eta-luxAB* fusion, with a 5-fold decrease in the levels of expression compared to growth in 0.3 M NaCl (Fig. 4b lower panel). Interestingly, sucrose repressed the expression of the *eta-luxAB* fusion to a similar extent in both $Agr^+$ and $Agr^-$ strains. Similar results were obtained with the *eta-xylE* fusion when grown under similar conditions for up to 18 h (data not shown). These results suggest a role for growth medium osmolarity in the regulation of an *agr*-dependent gene. Furthermore this would suggest that the expression of *eta* is more sensitive to the osmolyte sucrose than to NaCl and that this sensitivity becomes more pronounced in stationary phase cultures.



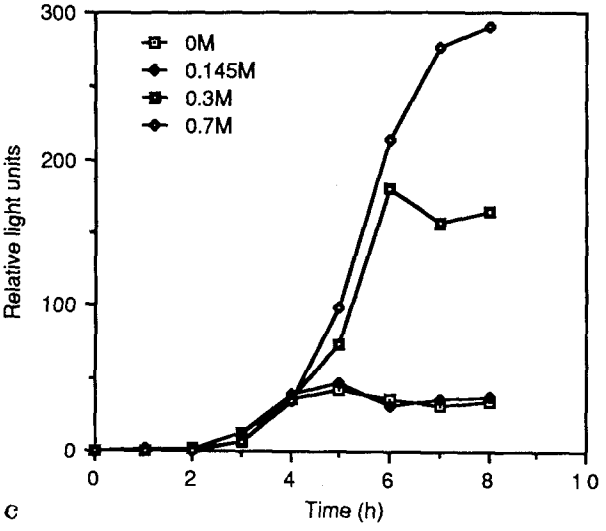
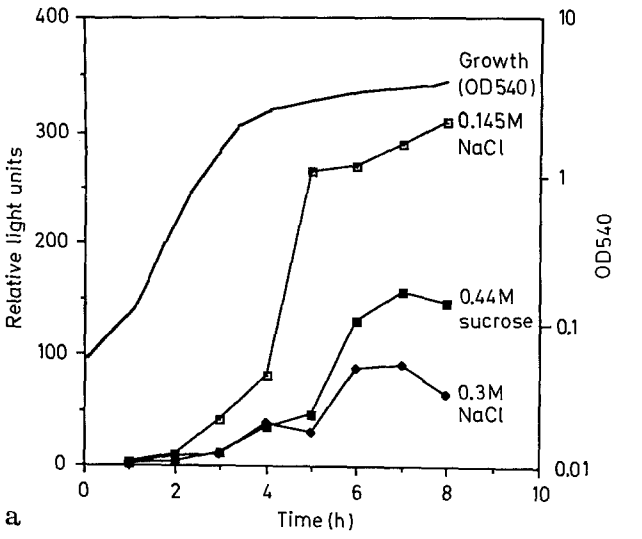




**Fig. 3a–c.** Osmoregulation of *eta* expression. **a** Effect of increasing NaCl concentration on the expression of *eta-luxAB* in 8325-4 ($Agr^+$); **b** effect of increasing osmolarity on the expression of pXL2 (*eta-xylE*) in both 8325-4 and ISP546. The specific activity of catechol 2,3-dioxygenase production was calculated where 1 milliunit corresponds to the formation of 1 mmol of 2-hydroxymuconic semialdehyde per min at 37° C. **c** Effect of increasing NaCl concentration on the expression of pKSB7 in the $Agr^+$ strain 8325-4



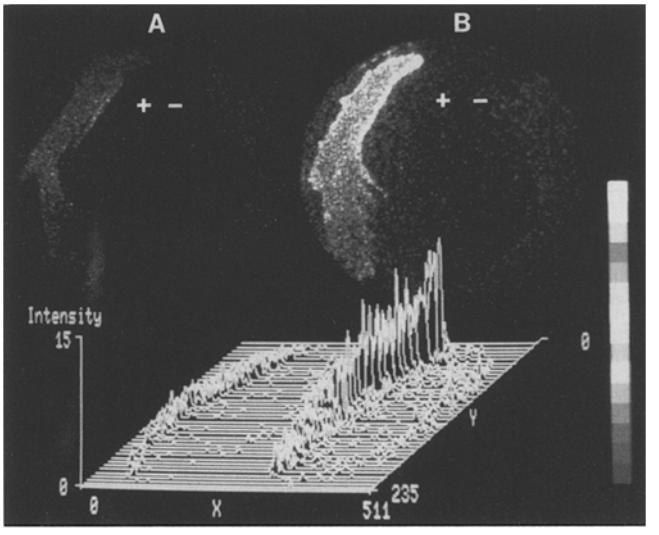




**Fig. 4a, b.** Comparison of 0.3 M NaCl and 0.44 M sucrose as osmotic repressors of *eta* expression. **a** Effect of sucrose (0.44 M) and NaCl (0.3 M) on *eta-luxAB* expression in liquid culture up to 8 h post-inoculation. **b** Levels of light production from both 8325-4 and ISP546 containing pSP100 when grown for 18 h on agar containing sucrose to 0.44 M (A) and NaCl to 0.3 M (B). Relative light production from both the $Agr^+$ and $Agr^-$ strains was calculated using a Hamamatsu Argus 100 Vim 3 photon video camera and was for 0.3 M NaCl: $Agr^+$, 28695; $Agr^-$, 2834; and for 0.44 M sucrose: $Agr^+$, 5242; $Agr^-$, 583 relative light units

### *The effect of DNA supercoiling on* eta *promoter activity*

Two independent approaches were taken to study the effect of DNA supercoiling (changes in DNA topology) on *agr*-regulated gene expression in *S. aureus*. The first used the antibiotic novobiocin to inhibit DNA gyrase, an enzyme responsible for adding negative supercoils to DNA in an ATP-dependent fashion (Sutcliffe et al. 1989). Novobiocin competitively inhibits the binding to ATP by DNA gyrase, resulting in an overall relaxation of the cellular DNA. The second approach involved the

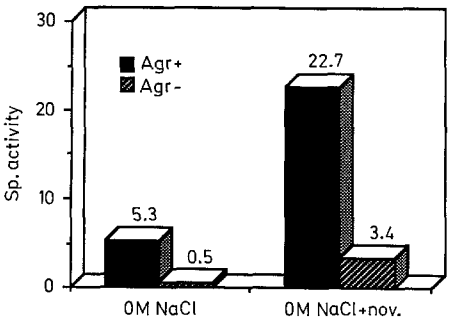



**Fig. 5.** Effect of novobiocin on stationary phase expression of the *eta-xylE* fusion in *S. aureus* strains 8325-4 ($agr^+$) and ISP546 ($Agr^-$). The specific activities of the respective strains are given by the *number* at the top of each column

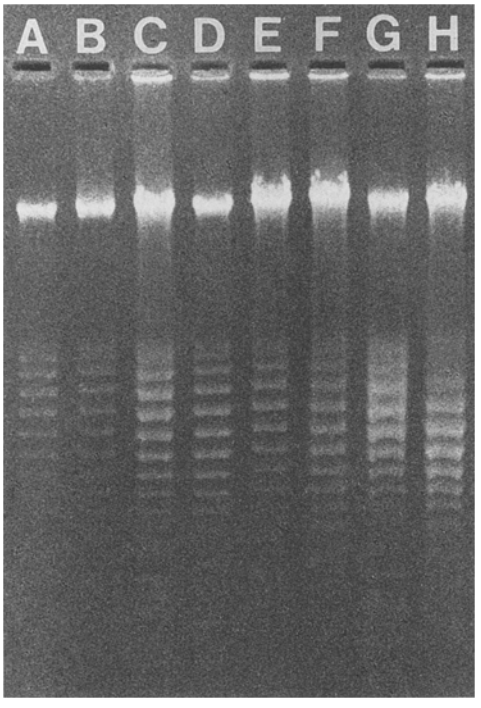



**Fig. 6.** The effect of osmolarity on in vivo plasmid supercoiling. Plasmid DNA was isolated from strains grown under conditions of varying NaCl concentration. Topoisomers were resolved by electrophoresis in 1% agarose gel containing chloroquine (25 µg/ml). Under these conditions the more negatively supercoiled topoisomers migrate more slowly through the gel. Topoisomer distribution of plasmid DNA isolated from $Agr^+$ and $Agr^-$ strains, respectively, when grown in TSB containing: lanes A and B, no added NaCl; lanes C and D, topoisomer 0.145 M NaCl; lanes E and F, 0.3 M NaCl; lanes G and H, 0.7 M NaCl

use of plasmids as indicators of changes in the level of DNA supercoiling.

*S. aureus* strains 8325-4 and ISP546 containing plasmid pXL2 were grown in the presence of sub-inhibitory concentrations of novobiocin (500 pg/ml) for 18 h. Growth in novobiocin resulted in a significant increase in the activity of the *eta-xylE* fusion in both the $Agr^+$ and $Agr^-$ host strains (Fig. 5). The increase was of approximately the same order in both strains ($Agr^+$, 4.2-fold; $Agr^-$, 6.8-fold) and suggests that the novobiocin-induced DNA relaxation enhances expression from the *eta* promoter. That this increase in expression is of the same order of magnitude in both $Agr^+$ and $Agr^-$ strains suggests that the *agr* locus itself is insensitive to novobiocin-induced changes in DNA topology.

To study the effect of osmolarity on DNA topology in *S. aureus*, plasmids were used as indicators of changes in the level of DNA supercoiling (Balke and Gralla 1987; Dorman et al. 1988). pBS1, a derivative of the staphylococcal plasmid pCW59 (Wilson et al. 1981), was isolated which lacked the smaller 2.3 kb *Hin*dIII fragment containing the tetracycline gene. Strains 8325-4 ($Agr^+$) and ISP546 ($Agr^-$) containing the plasmid pBS1 were grown in TSB containing varying concentrations of NaCl. After 18 h growth the cells were harvested, plasmid DNA isolated and the plasmid topoisomers were resolved by electrophoresis in an agarose gel containing chloroquine. Figure 6 shows the distribution of topoisomers isolated from $Agr^+$ and $Agr^-$ hosts grown in differing concentrations of NaCl. Plasmid DNA isolated from *S. aureus* does not show the same degree of sensitivity to changes in the extracellular salt concentration as is observed in *E. coli* and *Salmonella typhimurium* (Higgins et al. 1988). In *S. aureus* a small change in topoisomer distribution towards more negatively supercoiled DNA occurs in plasmids isolated from cells grown in TSB containing 0.7 M NaCl (Fig. 6, lane G). Interestingly this shift in topoisomer distribution is not observed in plasmids isolated from the $Agr^-$ strain grown in the same conditions (Fig. 6, lane H).

## Discussion

The discovery that the expression of bacterial virulence genes is regulated in a coordinated manner in response to environmental stimuli has been a major advance in understanding the control of pathogenic processes. Insights into the molecular nature of these regulatory processes are founded primarily on results obtained in gram-negative systems (Miller et al. 1989). *S. aureus* is an excellent candidate for testing the relevance of coordinated control motifs to a gram-positive organism. The present study has concentrated on the environmental regulation of one *S. aureus* virulence gene, *eta*. Its expression was demonstrated to be sensitive to changes in growth medium osmolarity, to changes in growth phase and to treatment with a DNA gyrase-inhibiting drug (novobiocin), and increases in its expression were shown to correlate with a relaxation of bacterial DNA supercoiling.

Previous work has shown that the expression of the *eta* gene is dependent on *agr*, the locus encoding the Accessory Gene Regulator (O'Toole and Foster 1987). Results obtained in this study with the *lux* and *xylE* reporter gene systems show that the Agr sensitivity is maintained when the *eta* promoter is carried on multicopy plasmids. Moreover, the previously described growth phase-dependent expression of *eta* is also reproduced with both reporter systems, demonstrating that the expression of the *eta* promoter on multicopy plas-

mids is modulated by the environment in a manner approximating to that seen when it is on the chromosome.

*S. aureus* is a halotolerant bacterium and can be isolated from environments that exert a high level of osmotic stress. However, this organism is also capable of growth in low osmotic conditions, demonstrating an ability to adapt to either type of environment. In gram-negative pathogens, adaptation to osmotic change forms part of the genetic switch controlling virulence gene expression (Bernardini et al. 1990; Chatfield et al. 1991; Dorman et al. 1989; Galan and Curtiss 1990). Experiments with strains of *S. aureus* harbouring either *eta-luxAB* or *eta-xylE* recombinant plasmids show that expression of the epidermolytic toxin A gene is osmotically regulated and, moreover, that Agr is involved in the transmission of the osmotic signal to the *eta* promoter. Expression of the *eta* fusions was inversely related to osmolyte levels and similar results were obtained with two molecularly distinct osmolytes, NaCl and sucrose. However, *eta* expression was more readily repressed by sucrose than by NaCl. This could reflect a specific adaptation on the part of *S. aureus* to NaCl as an inducer of osmotic stress, perhaps because NaCl is a common component of the habitat of this bacterium. This finding has important implications for our understanding of *S. aureus* physiology and for the efficacy of measures used to exclude this bacterium from economically important substrates (e.g. food) where spoilage or toxin contamination is undesirable.

The dependence of *eta* expression on environmental factors (osmolarity and growth phase) and on a member of the histidine protein kinase/response regulator family of signal transduction systems (Agr) is reminiscent of several environmentally controlled genes in gram-negative bacteria with "house-keeping" and/or virulence roles (Stock et al. 1989). Expression of many of these genes has been shown to be sensitive to changes in DNA topology (including DNA supercoiling) (Dorman 1991). The *eta* promoter is strongly induced in cultures of *S. aureus* grown in the presence of sub-inhibitory concentrations of the DNA gyrase-inhibiting drug, novobiocin. This suggests that relaxation of DNA favours expression of this gene, a situation that has been described for some genes in gram-negative bacteria, including *gyrA* and *gyrB* (encoding the A and B subunits of DNA gyrase, respectively, in *E. coli*; Menzel and Gellert 1987) and *tonB*, (encoding the periplasm-bridging protein TonB, involved in iron and vitamin transport in *E. coli* and *S. typhimurium*; Dorman et al. 1988). The genes coding for GyrA and GyrB in *S. aureus* have recently been cloned and sequenced and the inferred amino acid sequences are highly homologous to those of the enzyme from *E. coli* (Hopewell et al. 1990). Thus, it is highly probable that novobiocin affects DNA gyrase in *S. aureus* in a manner similar to that by which it inactivates the *E. coli* enzyme. The hypersensitivity to novobiocin, which is characteristic of *S. aureus*, probably reflects an enhanced ability on the part of the drug to enter this gram-positive bacterium in the absence of a protective outer membrane, although this has not been formally proven.

The ability of environmental factors to influence DNA supercoiling levels is thought to be crucial in the transmission of environmental signals to the transcription machinery of the cell (Dorman 1991; Drlica 1990). The apparent sensitivity of *eta* to novobiocin treatment, its membership of the Agr regulon, and sensitivity to changes in osmolarity transmitted via Agr are highly significant. These observations together with the finding that *S. aureus* varies the linking number of reporter plasmids in a manner analogous to that previously described in enteric bacteria strongly suggest that global control of gene expression in *S. aureus* is managed through a mechanism approximating to that obtained in *E. coli* and *S. typhimurium*. Significantly, high osmolarity (0.7 M NaCl) results in an increase in negative supercoiling of plasmid DNA, correlating with the negative effects of both osmolarity and negative supercoiling (*eta* is induced by the DNA gyrase inhibitor, novobiocin) on the expression of reporter gene fusions to the *eta* promoter.

The shift in reporter plasmid supercoiling observed at 0.7 M NaCl was not observed in the $Agr^-$ strain ISP546. Importantly, the reporter plasmid used in these studies, pBS1, carries no *agr*-dependent genes and carries no known binding site for putative *agr*-encoded functions (unpublished data). Thus the effect is unlikely to be due to a direct effect on plasmid topology by *agr* acting *in trans*. This is of particular importance given the ability of plasmid gene expression to influence levels of both positive and negative supercoiling of the plasmids themselves (Drlica 1990). These transcription-induced variations in topology are quickly reset in cells that are wild-type for both DNA topoisomerase I and DNA gyrase and the cells used in this study are not deficient in these functions. Therefore, transcriptional effects on plasmid supercoiling can probably be disregarded, suggesting that Agr may influence plasmid supercoding by altering the activity of DNA gyrase, at least under conditions of osmotic stress. Whether this is a direct affect or is part of a cascade is currently unknown. Thus, in addition to specific effects on gene expression within the Agr regulon, Agr could play a more general role in the global control of *S. aureus* DNA-dependent processes. The finding that *agr* mutants are fully viable argues against either its specific or ancillary roles being essential to the cell.

The emerging picture of *eta* is of a gene possessing a promoter under multifactorial control, with a requirement for the Agr system for maximal expression and an Agr-independent sensitivity to changes in DNA supercoiling. Thus it shares features characteristic of virulence genes in gram-negative bacteria, many of which are regulated by a hierarchy of control elements. Here sensitivity to changes in DNA supercoiling offers a crude, general control mechanism upon which more refined and specific control elements are imposed. This interplay of regulatory mechanisms is particularly evident from the data presented in Fig. 3b. At high osmolarity the expression of the fusion product in the $Agr^+$ strain is observed to drop below that observed in the $Agr^-$ strain. As the *agr* locus encodes no known repres-

sor of gene activity, this may appear surprising. However if the repressive effect of increased negative supercoiling (observed to occur at high osmolarity; Fig. 6) is superimposed on the repressive effects of high osmotic stress (*agr* dependent) then a situation may result where *eta* promoter activity is repressed below the basal level.

Studies of global control circuits involved in regulating gene expression in gram-positive bacteria are currently at an early stage. The work described here shows that one member of the Agr regulon in *S. aureus*, the *eta* gene, is regulated by a control hierarchy having several features in common with the well studied gram-negative systems. These findings provide an excellent tool for elucidating the molecular basis of global gene regulation in this gram-positive pathogen.

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## References

Ahmed KA, Stewart GSAB (1988) Cloning of the *lux* genes into *Lactobacillus casei* and *Streptococcus lactis*: phosphate-dependent light production. Biochem Soc Trans 16:1068

Ahmed KA, Stewart GSAB (1991) The production of bioluminescent lactic acid bacteria suitable for the rapid assessment of starter culture activity in milk. J Appl Bacteriol 70:113–120

Balke V, Gralla J (1987) Changes in the linking number of supercoiled DNA accompany growth transitions in *E. coli*. J Bacteriol 169:4499–4506

Bayles KW, Iandolo JJ (1989) Genetic and molecular analysis of the gene encoding staphylococcal enterotoxin D. J Bacteriol 171:4799–4806

Bernardini ML, Fontaine A, Sansonetti PJ (1990) The two component regulatory system OmpR-EnvZ controls the virulence of *Shigella flexneri*. J Bacteriol 172:6274–6281

Bjorklind A, Arvidson S (1980) Mutants of *Staphylococcus aureus* affected in the regulation of exoprotein synthesis. FEMS Lett 7:203–206

Chatfield SN, Dorman CJ, Hayward C, Dougan G (1991) Role of *ompR* dependent genes in *Salmonella typhimurium* virulence: mutants deficient in OmpC and OmpF are attenuated *in vivo*. Infect Immun 59:449–452

Dorman CJ (1991) DNA supercoiling and environment regulation of gene expression in pathogenic bacteria. Infect Immun 59:745–749

Dorman CJ, Barr GC, Ni Bhriain N, Higgins CF (1988) DNA supercoiling and the anaerobic and growth phase regulation of *tonB* expression. J Bacteriol 170:2816–2826

Dorman CJ, Chatfield S, Higgins CF, Hayward C, Dougan G (1989) Characterisation of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated *in vivo*. Infect Immun 57:2136–2140

Drlica K (1990) Bacterial topoisomerases and the control of DNA supercoiling. Trends Genet 6:433–437

Easmon CSF, Adlam C (eds) (1983) Staphylococci and staphylococcal infections. vols 1 and 2. Academic Press, London

Galan JE, Curtiss R III (1990) Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. Infect Immun 58:1879–1885

Gaskill ME, Khan SA (1988) Regulation of enterotoxin B gene in *Staphylococcus aureus*. J Biol Chem 263:6276–6280

Gasson MJ, Anderson PH (1985) High copy number plasmid vectors for use in lactic streptococci. FEMS Lett 30:193–196

Higgins CF, Dorman CJ, Stirling DA, Waddell L, Booth IR, May G, Bremer E (1988) A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *Salmonella typhimurium* and *E. coli*. Cell 52:569–584

Hopewell R, Oram M, Briesewitz R, Fischer LM (1990) DNA cloning and organisation of the *Staphylococcus aureus gyrA* and *gyrB* genes: close homology among proteins and implications for 4-quinolone action and resistance. J Bacteriol 172:3481–3484

Janzon L, Arvidson S (1990) The role of the δ-lysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) of *Staphylococcus aureus*. EMBO J 9:1391–1399

Janzon L, Lofdahl S, Arvidson S (1986) Evidence for a coordinate transcriptional control of alpha-toxin and protein A synthesis in *Staphylococcus aureus*. FEMS Lett 33:193–198

Janzon L, Lofdahl S, Arvidson S (1989) Identification and nucleotide sequence of the delta-lysin gene, *hld*, adjacent to the accessory gene regulator (*agr*) of *Staphylococcus aureus*. Mol Gen Genet 219:480–485

Kreiswirth B, Lofdahl S, Betley MJ, O'Reilly M, Schlievert PM, Bergdoll MS, Novick RP (1983) The toxic shock syndrome exotoxin structural gene is not transmitted by a prophage. Nature 305:709–712

Lee CY, Schmidt JJ, Johnson-Winegar AD, Spero L, Iandolo JJ (1987) Sequence determination and comparison of the exfoliative toxin A and toxin B genes from *Staphylococcus aureus*. J Bacteriol 169:3904–3909

Mahmood R, Khan SA (1990) Role of upstream sequences in the expression of the staphylococcal enterotoxin B gene. J Biol Chem 2655:4652–4656

Mallonee DH, Glatz BA, Pattee PA (1982) Chromosomal mapping of a gene affecting enterotoxin A production in *Staphylococcus aureus*. Appl Environ Microbiol 43:397–402

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory Press, Cold Spring Habour, New York

Meighen EA (1988) Enzymes and genes from the *lux* operons of bioluminescent bacteria. Annu Rev Microbiol 42:151–176

Menzel R, Gellert M (1987) Fusions of the *Escherichia coli gyrA* and *gyrB* control regions to the galactokinase gene are inducible by coumermycin treatment. J Bacteriol 169:1272–1278

Miller JF, Mekalanos JJ, Falkow S (1989) Coordinate regulation and sensory transduction in the control of bacterial virulence. Science 243:916–922

Morfeldt T, Janzon L, Arvidson S, Lofdahl S (1988) Cloning of a chromosomal locus (*exp*) which regulates the expression of several exoprotein genes in *Staphylococcus aureus*. Mol Gen Genet 211:435–440

Novick RP (1963) Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. Virology 33:155–166

Oskouian B, Stewart GC (1990) Repression and catabolite repression of the lactose operon of *Staphylococcus aureus*. J Bacteriol 172:3804–3812

Peng HI, Novick RP, Kreiswirth B, Kornblum J, Schlievert P (1988) Cloning, characterisation and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. J Bacteriol 170:4365–4372

Ray C, Hay RE, Carter HL, Moran CP (1985) Mutations that affect utilisation of a promoter in stationary-phase *Bacillus subtilis*. J Bacteriol 163:610–614

Rescei P, Kreiswirth B, O'Reilly M, Schlievert P, Gruss A, Novick RP (1986) Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr*. Mol Gen Genet 202:58–61

Ronson CW, Nixon BT, Ausubel FM (1987) Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell 49:579–581

Sakuri S, Suzuki H, Kondo I (1988) DNA sequencing of the *eta* gene coding for staphylococcal exfoliative toxin serotype A. J Gen Microbiol 134:711–717

Stock JB, Ninfa AJ, Stock AM (1989) Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol Rev 53:450–490

Stock JB, Stock AM, Mottonen J (1990) Signal transduction in bacteria. Nature 344:395–400

Sutcliffe JA, Gootz TD, Barrett JF (1989) Biochemical characteristics and physiological significance of major DNA topoisomerases. Antimicrob Agents Chemother 33:2027–2033

O'Toole P, Foster TJ (1986) Molecular cloning and expression of the epidermolytic toxin A gene of *Staphylococcus aureus*. Microbial Pathogenesis 1:583–594

O'Toole P, Foster TJ (1987) Nucleotide sequence of the epidermolytic toxin A gene of *Staphylococcus aureus*. J Bacteriol 169:3910–3915

Wilson C, Skinner S, Shaw W (1981) Analysis of two chloramphenicol resistance plasmids from *Staphylococcus aureus*: Insertional inactivation of chloramphenicol resistance, mapping of restriction sites, and construction of cloning vehicles. Plasmid 5:245–258

Yanisch-Perron C, Vieira J, Messing J (1983) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC vectors. Gene 33:103–119

Zukowski MM, Gaffney DF, Speck D, Kauffmann M, Findeli A, Wisecup A, Lecocq J (1983) Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on expression of a cloned *Pseudomonas* gene. Proc Natl Acad Sci USA 80:1101–1105

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