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The *bacA* gene, which determines bacitracin susceptibility in *Streptococcus pneumoniae* and *Staphylococcus aureus*, is also required for virulence

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Homologues of *Escherichia coli bacA*, encoding extremely hydrophobic proteins, were identified in the genomes of *Staphylococcus aureus* and *Streptococcus pneumoniae*. Allelic replacement mutagenesis demonstrated that the gene is not essential for *in vitro* growth in either organism, and the mutants showed no significant changes in growth rate or morphology. The *Staph. aureus bacA* mutant showed slightly reduced virulence in a mouse model of infection and an eightfold increase in bacitracin susceptibility. However, a *Strep. pneumoniae bacA* mutant was highly attenuated in a mouse model of infection, and demonstrated an increase in susceptibility to bacitracin of up to 160000-fold. These observations are consistent with the previously proposed role of BacA protein as undecaprenol kinase.

Keywords: bacitracin, *bacA*, undecaprenol kinase, *Staphylococcus aureus*, *Streptococcus pneumoniae*

INTRODUCTION

Undecaprenol monophosphate (UP or C_{55}-P) is a key component of cell wall biosynthesis. The precursor for UP, undecaprenol pyrophosphate (UPP), is synthesized by UPP synthetase from five-carbon isopentenyl pyrophosphate units (Apfel *et al*., 1999; Shimizu *et al*., 1998). In order to participate in cell wall biosynthesis, newly synthesized UPP is dephosphorylated by a membrane-bound pyrophosphatase to UP (Goldman & Strominger, 1972), which locates to the cell membrane where it functions as a lipid carrier for sugar-peptide units in peptidoglycan and, in Gram-positive bacteria, teichoic acid biosynthesis (Storm & Strominger, 1973; Baddiley, 1973; Reusch, 1984; Navarre & Schneewind, 1999). Following completion of the teichoic acid biosynthetic cycle the UP carrier is released for reuse; but synthesis of peptidoglycan monomer units and their assembly by transglycosylases to form mature peptidoglycan results in the release into the periplasm of UPP, which must be converted back to UP by the pyrophosphatase.

The macrocyclic dodecapeptide bacitracin is a useful tool for exploring C_{55} lipid recycling in bacteria. This antibiotic has been available to clinical practitioners for many years but has not been used systemically since the 1950s, when it was found to be nephrotoxic at therapeutic levels and, on rare occasions, to induce anaphylactic shock. Its primary use today is to treat skin and surface tissue infections such as impetigo or suppurative conjunctivitis, usually in combination with polymyxin B and neomycin (Kanof, 1970). The principal mode of action of bacitracin is to bind tightly to UPP in the presence of a bound divalent metal cation, sequestering UPP and preventing its interaction with phosphatase (Stone & Strominger, 1971, 1972; Storm & Strominger, 1973; Drablos *et al*., 1999). This in turn prevents the return of UP lipid carrier to the cycle, thereby inhibiting peptidoglycan and teichoic acid biosynthesis (Anderson *et al*., 1972; Toscano & Storm, 1982).

Despite its importance, the total amount of UP in cells is

Abbreviations: PN, pyelonephritis; RTI, respiratory tract infection; UP, undecaprenol monophosphate; UPP, undecaprenol pyrophosphate.

The GenBank accession number for the sequence reported in this paper is AF228662.

low and in cell-free preparations is limiting for the synthesis of both polymers (Baddiley, 1973). It has been suggested that UP availability controls the rate of cell wall synthesis (Anderson *et al*., 1972; Baddiley, 1973), so it is interesting that a further source of UP is provided by a membrane-bound undecaprenol phosphokinase activity which reversibly converts the C_{55} alcohol undecaprenol into UP (Higashi *et al*., 1970a; EC 2.7.1.66). As the majority of membrane-associated undecaprenol is present in the free alcohol form – approximately 90% in both *Staphylococcus aureus* and *Streptococcus pyogenes* (Higashi *et al*., 1970b; Reusch & Panos, 1976), 80% in late exponential phase *Streptococcus faecalis* (Umbreit *et al*., 1972) and 25% in exponential phase *Lactobacillus plantarum*, increasing to 73% in stationary phase cells (Thorne, 1973) – it appears that this apparently futile reaction has the capacity to play an important role in UP availability and regulation of cell wall biosynthesis.

The gene encoding undecaprenol kinase has not been definitively identified, but an *Escherichia coli* strain carrying the *bacA* gene on a multicopy plasmid displayed an increase in membrane-associated isoprenol kinase activity and reduced susceptibility to bacitracin without a concomitant increase in UPP synthetase activity (Cain *et al*., 1993). It was therefore hypothesized that *bacA* encodes undecaprenol kinase, and that when this enzyme is overproduced it is able to produce sufficient supplies of UP from undecaprenol to overcome the effects of UPP sequestration, thereby causing bacitracin resistance. However, Gram-negative bacteria are generally insensitive to bacitracin and, in the absence of the membrane-permeabilizing agent polymyxin B, the growth of *E. coli* is unaffected up to bacitracin concentrations of 200 μg ml^{-1} (Toscano & Storm, 1982; Cornelissen & Van den Bossche, 1983). Hence very high concentrations of bacitracin were used in this study, and it is possible that under these conditions the observed response to bacitracin reflects alternative or additional effects, such as non-specific toxicity or membrane permeabilization. We therefore wished to characterize the role of the *bacA* gene in bacitracin-susceptible Gram-positive organisms. Here we describe the identification of the *bacA* genes of the Gram-positive organisms *Staph. aureus* and *Streptococcus pneumoniae*, and examine the effect of *bacA* mutagenesis on viability, virulence and bacitracin susceptibility.

METHODS

Bacterial strains and plasmids. *Strep. pneumoniae* 0100993 (NCIMB 40794; serotype 3) is a plasmid-free, encapsulated clinical isolate kindly provided by D. Felmington (University College Hospital, London, UK). *Staph. aureus* RN4220 is a restriction-deficient 8325-4 derivative (Kreiswirth *et al*., 1983). *Staph. aureus* WCUH29 is an encapsulated pathogenic strain (NCIMB 40771) kindly provided by V. Hryniewicz (Children's University Hospital, Warsaw, Poland). pBluescriptTet is a derivative of pBluescript which does not replicate in *Staph. aureus* and contains the *tetK* gene from pT181 (Khan & Novick, 1983).

Generation of *Staph. aureus* allelic replacement mutants. A 630 bp upstream and 518 bp downstream region of *Staph. aureus bacA* were amplified using PCR and sequentially cloned either side of an erythromycin-resistance gene, *ermC*, from pE194 (Horinouchi & Weisblum, 1982) in pBluescriptTet in *E. coli* DH10B. The resulting allelic replacement construct was introduced into *Staph. aureus* RN4220 by electroporation, and erythromycin- and tetracycline-resistant (Erm^R Tet^R) colonies were picked and confirmed as cointegrants using diagnostic PCR. In the latter, DNA primers designed to hybridize within *ermC* were paired with primers hybridizing to distal chromosomal sequences to generate DNA products of characteristic size. ϕ11 transducing lysates (Novick, 1991) were prepared from cointegrants and used to transduce *Staph. aureus* RN4220. Erm^R transductants were selected and screened for tetracycline sensitivity (Tet^S), and Erm^R Tet^s clones were examined by diagnostic PCR and Southern blot analysis to confirm that the expected 898 bp deletion had occurred. The *bacA* mutation was ϕ11 transduced into the pathogenic *Staph. aureus* strain WCUH29, and the resulting Erm^R clone was reconfirmed as described above. Sensitivity to osmotic stress was determined by plating *Staph. aureus* strains on Bacto Staphylococcus medium 110 (Chapman, 1945) and incubating for 12 h at 37 °C.

Generation of *Strep. pneumoniae* allelic replacement mutants. A 329 bp upstream and 402 bp downstream region of *bacA* were PCR-amplified from *Strep. pneumoniae* 0100993 chromosomal DNA, extracted and purified as previously described (Paton, 1996). The fragments were used to make a construct in which they flanked an erythromycin-resistance gene, *ermAM*, from pAMβ1 (Martin *et al*., 1987), which was used to transform *Strep. pneumoniae* 0100993 competent cells prepared according to standard protocols. Cells (10^6) were incubated with 1–5 μg allelic replacement construct at 30 °C for 40 min in AGCH medium (Lacks, 1966) supplemented with 0·2% sucrose, 1 mM $CaCl_2$ and 1·7 μg competence stimulating peptide CSP-1 ml^{-1} (Havarstein *et al*., 1995), then transferred to 37 °C for 70 min. The transformation mix was incubated for a further 20 min with 0·1 mg erythromycin ml^{-1}; then for 10 min at 37 °C with 10% glycerol; and finally for 10 min on ice. The mix was plated in AGCH medium containing 0·3% sucrose, 0·2% yeast extract, 1% agar and 1 μg erythromycin ml^{-1}, and incubated at 37 °C for 36 h in 5% CO_2. Erm^R colonies were picked and grown overnight in Todd–Hewitt broth (THB) supplemented with 0·5% yeast extract. Chromosomal DNA from *Strep. pneumoniae* 0100993 Erm^R clones was examined using Southern blot analysis and diagnostic PCR to verify that the expected 704 bp chromosomal deletion had occurred.

MIC determination. Antimicrobial agents used in the study were obtained from Sigma. Bacitracin stock solution was made up at 2048 μg ml^{-1} in 5% DMSO. MICs were determined using the NCCLS recommended procedure for broth microdilution. *Strep. pneumoniae* strains were tested in cation-adjusted Mueller–Hinton broth (MHB) supplemented with 5% lysed horse blood, while *Staph. aureus* strains were tested in cation-adjusted MHB. *Strep. pneumoniae* MICs for bacitracin were also determined in THB with 5% yeast extract, supplemented where appropriate with 5% horse serum.

Mouse pyelonephritis (PN) infection using *Staph. aureus*. Overnight cultures of *Staph. aureus* WCUH29 or isogenic mutant strains grown at 37 °C were washed twice in sterile PBS and diluted to an OD_{600} of 0·2. Note that all optical density measurements on microbial samples were taken with a

Beckman DU520 spectrophotometer using 1 cm path length cuvettes.

For each strain used, five female CD-1 mice (18–20 g) were inoculated with 0·2 ml suspension (10^7 bacteria) by tail vein injection. Mice were monitored twice daily for signs of illness and any which appeared moribund were killed prior to the end of the experiment. All animals were killed via CO_2 overdose at 5 d post-inoculation. Both kidneys were removed using aseptic techniques and homogenized in 1 ml PBS. Viable bacteria were enumerated after plating on tryptic soy agar (TSA) plates. The significance of attenuation in the mutant group compared to the wild-type was calculated by *t*-test analysis using a two-sample equal variance (type 2) and a one-tailed distribution.

Mouse respiratory tract infection (RTI) model using *Strep. pneumoniae*. TSA plates containing 5% sheep blood were inoculated from frozen stocks of *Strep. pneumoniae* 0100993 or isogenic mutant strains and grown overnight at 37 °C in 5% CO_2. Bacteria were recovered from the plates and resuspended in PBS to an OD_{600} of 0·8–1·0 (10^7–10^8 bacteria ml^{-1}). For each strain used, five male CBA/J mice (14–16 g) were anaesthetized with 3% isoflurane, and 50 µl of the prepared inoculum was administered by intranasal instillation. The mice were allowed to recover and given food and water *ad libitum*. Animals were observed three times daily and moribund animals, or those showing signs of cyanosis, hypothermia or staring coat, were killed by CO_2 overdose. Surviving animals were killed 48 h post-infection. The lungs were removed aseptically, homogenized in 1 ml PBS, and enumerated for viable bacteria. The significance of attenuation in the mutant group compared to the wild-type was calculated by *t*-test analysis as described above.

RESULTS

Identification of *bacA* homologues in *Staph. aureus* and *Strep. pneumoniae*

A *Strep. pneumoniae* homologue of the 273 aa *E. coli* BacA protein (Cain *et al.*, 1993) was identified in translation on contig 81 of *Strep. pneumoniae* type 4 sequence data obtained from The Institute for Genomic Research (http://www.tigr.org), using BLAST version 2.0.4 software (Altschul *et al.*, 1997). The probable translation start site was chosen by homology and for optimal spacing from the potential ribosome-binding site (Gold *et al.*, 1981). The ORF encodes a putative protein of 281 aa. A potential promoter sequence conforming to the *E. coli* σ^{70} promoter consensus at the −10 and −35 regions was identified 15 bp upstream (Sabelnikov *et al.*, 1995) and the up- and downstream genes, which encode a protein with low homology to invertase proteins and an *E. coli dinX* homologue, respectively, are both transcribed in the opposite orientation to *bacA*. A compound BOX element was identified 35 bp downstream of the gene. These repetitive elements are found throughout the *Strep. pneumoniae* genome, but their function is unknown (Martin *et al.*, 1992).

A *Staph. aureus* ORF which in translation encodes a 291 aa BacA homologue was identified in library sequence from the pathogenic *Staph. aureus* strain WCUH29, and the sequence has been submitted to GenBank under accession number AF228662. The gene is preceded by a divergently transcribed *cydD* transporter homologue. The downstream ORF encodes a lysine-rich protein of uncertain function, but as it is separated from the *bacA* gene by 120 bp of intervening DNA it seems unlikely that the two are transcriptionally linked.

Alignment of the *Strep. pneumoniae* and *Staph. aureus* BacA-like protein sequences with other BacA homologues demonstrated that the sequences range from 20 to 51% identical at the protein level. There is particular conservation in two regions, Ile^{10}–Leu^{32} and Ile^{160}–Ile^{179} (*Strep. pneumoniae* numbering) as shown in Fig. 1. The *Staph. aureus*, *Strep. pneumoniae* and *E. coli* proteins have very similar Kyte–Doolittle hydrophilicity plots (not shown) including eight potential *trans*-membrane hydrophobic segments 15–20 aa in length. Composition analysis of the three protein sequences showed that they each contain 50% hydrophobic amino acids, and that in each case isoleucine and leucine account for 20–25% of the amino acid residues.

Generation of *bacA* mutants of *Staph. aureus* and *Strep. pneumoniae*

Constructs for allelic replacement of the *bacA* homologues of *Staph. aureus* RN4220 and of the pathogenic strains *Staph. aureus* WCUH29 and *Strep. pneumoniae* 0100993 were made by PCR amplification. As described above, the *Strep. pneumoniae bacA* gene is monocistronic and the *Staph. aureus bacA* gene does not appear to be transcriptionally linked with its downstream gene, so it is extremely unlikely that gene replacement of either would have a polar effect on expression of neighbouring genes. However, in order to minimize potential polar effects, the PCR primers were chosen so that flanking genes and intergenic regions including potential promoters would remain intact in the deletion mutant. In addition, transcriptional termination signals were removed from the erythromycin-resistance gene marker (*erm*) in each case, and the cassettes were designed to integrate in the same orientation as the target gene in an attempt to ensure transcription of the downstream region.

The *Staph. aureus bacA* allelic replacement construct was transformed into *Staph. aureus* RN4220 and Erm^R Tet^R cointegrant colonies were obtained. ϕ11 transduction was used to resolve the cointegrant and generate Erm^R *bacA* allelic replacement mutants of *Staph. aureus* RN4220 and WCUH29 as described. Similarly, an Erm^R allelic replacement mutant of *Strep. pneumoniae* 0100993 was generated. The expected chromosomal rearrangements were confirmed using PCR and Southern blot analysis. Serial subcultures of the *bacA* mutants and their wild-type parent strains were subject to growth curve analysis and microscopy, and exponential-phase cultures were subject to flow cytometric analysis, but no differences in cell size, morphology or growth characteristics at equivalent time points including lag phase and growth rate were detectable (data not shown). It therefore appears that

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sabaca  10  IILGVVEGLTEFAPVSSTGHMIL  32 // 170  IGISQAVAMWPGFSRSGSTI  189
spbaca  10  IFFGIVEGITEWLPISSTGHLIL  32 // 160  IGLFQVLALLPGTSRSGATI  179
aabaca   7  VVLGIVEGISEFLPISSTGHLIL  29 // 142  IGVFQSIAVIPGVSRSGSTI  161
bbbaca   8  IILGIIQGITEFLPISSSGHLLL  30 // 152  MGLMQGLGALPGISRSGITI  171
bsyubb  10  AILGIVEGLTEYAPVSSTGHMII  32 // 161  VGLFQCLSLWPGFSRSGSTI  180
ecbaca  11  AILGVVEGLTEFLPVSSTGHMII  33 // 160  IGCFQCLALWPGFSRSGATI  179
fjbaca   7  IVLAVIEGITEFLPVSSTGHMII  29 // 141  IGLFQCIAMIPGVSRSGASI  160
mtbaca   7  IVLAAAQGLTEFLPVSSSGHLAI  29 // 153  VGIAQTLALVPGVSRSGSTI  172
              .:.  :*::*: *:**:**: :              * :.  ** **** :*  .
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Fig. 1. Two conserved regions in BacA homologues. The *Staph. aureus* and *Strep. pneumoniae* BacA homologues (labelled sabaca and spbaca, respectively) were aligned with homologues from *Aquifex aeolicus* (aabaca; Deckert *et al.*, 1998), *Borrelia burgdorferi* (bbbaca; Fraser *et al.*, 1997), *Bacillus subtilis* (bsyubb; Moszer *et al.*, 1995; Kunst *et al.*, 1997), *E. coli* (ecbaca; Cain *et al.*, 1993), *Flavobacterium johnsoniae* (fjbaca; M. J. Kempf & M. J. McBride, GenBank direct submission AAD50462) and *Mycobacterium tuberculosis* (mtbaca; Cole *et al.*, 1998) using CLUSTALX. Single fully conserved residues are in bold and indicated by an asterisk; residues which are fully conserved within a strongly related group as defined by CLUSTALX are indicated by a double dot; and residues which are fully conserved within a weakly related group are indicated by a single dot.

Table 1. MIC data in μg ml^{-1} for *Strep. pneumoniae* and *Staph. aureus bacA* mutant and parent strains

MICs were determined in MHB with appropriate supplementation as described, except where indicated otherwise. A dash indicates that no MIC was determined.

Compound	*Strep. pneumoniae* 0100993	*Strep. pneumoniae* 0100993 *bacA* mutant	*Staph. aureus* WCUH29	*Staph. aureus* WCUH29 *bacA* mutant	*Staph. aureus* RN4220	*Staph. aureus* RN4220 *bacA* mutant
Bacitracin	8	0·008	32	2	64	4
Bacitracin (THB)	8	0·00005	–	–	–	–
Amoxycillin	0·015	0·015	64	64	0·25	0·25
Ceftriaxone	⩽0·015	⩽0·015	16	16	2	2
Mupirocin	0·25	0·125	0·125	0·125	0·125	0·125
Vancomycin	0·25	0·5	1	0·5	0·5	1
Polymyxin B	>256	>256	64	64	64	64
Cefuroxime	0·015	0·015	8	8	0·5	0·5
Tetracycline	0·5	0·5	0·5	0·5	0·5	0·5
Chloramphenicol	4	8	>8	>8	8	8
Gentamicin	8	8	0·5	0·5	0·5	0·25
Kanamycin	64	32	16	16	2	4
Spectinomycin	>8	>8	>8	>8	>8	>8
Ciprofloxacin	2	2	0·5	0·25	0·5	0·25

the *bacA* gene is not required for the viability *in vitro* of either *Staph. aureus* or *Strep. pneumoniae*. The *Staph. aureus* WCUH29 and RN4220 *bacA* mutants and their parent strains, which are salt-tolerant (Vijaranakul *et al.*, 1997), were also plated on high osmotic strength medium containing 7·5% NaCl but showed no differences in growth.

Bacitracin susceptibility of mutants

The MICs of the *Staph. aureus* WCUH29 and RN4220 *bacA* mutants, the *Strep. pneumoniae* 0100993 *bacA* mutant and their parent strains for a panel of antibiotics including bacitracin were determined (Table 1). Both *Staph. aureus bacA* mutants showed a 16-fold increase in susceptibility to bacitracin relative to their parent strains. In the case of the *Strep. pneumoniae* 0100993 *bacA* mutant (but not the wild-type strain), it was noted that MICs were significantly lower than wild-type but varied over 5–6 doubling dilutions, depending on the type of media and the solvent used for bacitracin. The mutant gave an MIC for bacitracin of 0·008 μg ml^{-1} in MHB, representing a 1000-fold increase in susceptibility compared to the parent strain; whereas in THB, the mutant reproducibly gave an MIC of 0·00005 μg ml^{-1} both with and without 5% horse serum, representing a 160000-fold increase in susceptibility. No change in susceptibility was observed for other test compounds.

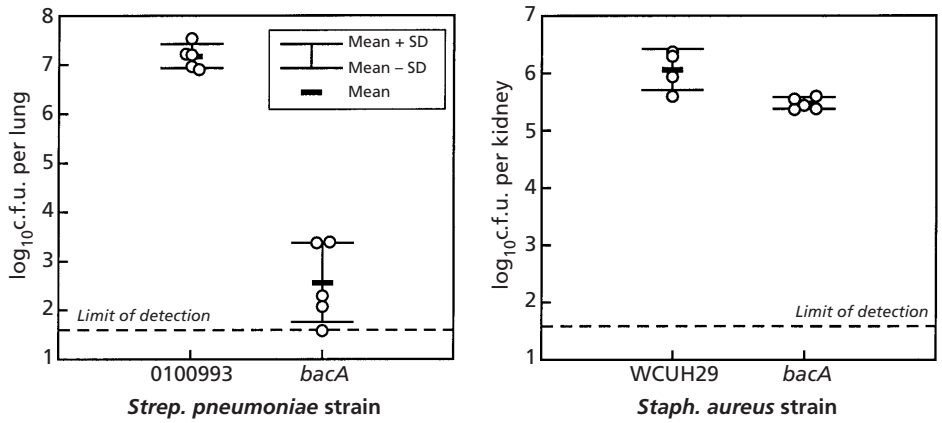

Fig. 2. *In vivo* analysis of *bacA* mutant strains. Infection models are mouse RTI for *Strep. pneumoniae*, and mouse haematogenous PN for *Staph. aureus*. For each model, the wild-type strain is designated by strain name and the mutant strain by *bacA*. Each mutant was tested in a five-animal group in comparison with a wild-type control group (one animal in the *Staph. aureus* WCUH29 control group died at 48 h).

Characterization of mutants in a mouse model

The *Strep. pneumoniae* 0100993 and *Staph. aureus* WCUH29 *bacA* mutants were tested in standard murine infection models (Fig. 2). Bacterial load in harvested tissue samples was enumerated as bacterial numbers in c.f.u. to directly reflect the capacity of the bacteria to grow and survive within the host, as this provides the most accurate assessment of attenuation. The *Strep. pneumoniae bacA* mutant was used to infect groups of mice intranasally using a standard murine RTI model, and the lungs were harvested 48 h post-infection. The *bacA* mutant group gave a mean bacterial count of 2·6 $\log_{10}$c.f.u. per lung, representing a 4·6 $\log_{10}$ attenuation in comparison with wild-type *Strep. pneumoniae* 0100993, which gave a mean count of 7·2 $\log_{10}$c.f.u. per lung. In a *Staph. aureus* mouse haematogenous PN infection model, the *Staph. aureus bacA* mutant showed a 0·6 log attenuation 5 d post-infection, giving a mean bacterial count of 5·5 $\log_{10}$c.f.u. per kidney as compared to 6·1 $\log_{10}$c.f.u. per kidney with the wild-type *Staph. aureus* WCUH29 infection control. *t*-test analysis showed that both mutants were significantly attenuated ($P \leqslant 0{\cdot}01$). Comparison of these data with the behaviour in these models of *Strep. pneumoniae* and *Staph. aureus* strains mutated in a variety of different genes affecting cell metabolism or virulence (data not shown) indicated that the *Strep. pneumoniae bacA* mutant is approaching the maximum attenuation of 6 logs which has been detected in the *Strep. pneumoniae* RTI model; while the *Staph. aureus bacA* mutant is only slightly impaired in comparison to the maximum attenuation we have detected in the *Staph. aureus* PN model, which is 2·5 logs.

DISCUSSION

Homologues of the probable *E. coli* undecaprenol kinase enzyme BacA are widespread among eubacteria. The *Strep. pneumoniae* and *Staph. aureus bacA* gene products described here are, respectively, 29 % and 43 % identical to the *E. coli bacA* gene product, and the unusually hydrophobic properties of these proteins are consistent with their proposed role as membrane-bound undecaprenol kinase enzymes. Although the size of purified *Staph. aureus* undecaprenol kinase enzyme was previously estimated by SDS-PAGE to be 17000 Da (Sandermann & Strominger, 1971, 1972) while the *Staph. aureus bacA* gene encodes a product of 32000 Da, both proteins are likely to behave anomalously during chromatographic purification and SDS-PAGE analysis due to their high hydrophobicity, and it is therefore entirely possible that they are one and the same. This is supported by protein composition analysis, as amino acid frequencies in the *Staph. aureus* undecaprenol kinase enzyme determined by physical methods (Sandermann & Strominger, 1971) are strikingly similar to those found in the *bacA* gene product. For example, the frequency of non-polar amino acids (P, A, V, I, M, L and F) estimated for the isolated enzyme following acid hydrolysis was 57·8 %, while the BacA protein described here contains 54·7 % non-polar residues.

Further evidence that the *bacA* gene product is involved in C_{55} lipid recycling is provided by the observation that the *Strep. pneumoniae bacA* mutant is hypersensitive to bacitracin, while its susceptibility to other cell-wall- and cell-membrane-targeting antibiotics, including amoxycillin, polymyxin B and vancomycin, remains unchanged. However, the MIC of the *Strep. pneumoniae bacA* mutant for bacitracin was found to be highly dependent on media and conditions. It has been reported that addition of 5 % sheep blood to MHB increases enterococcal MICs for bacitracin (Butaye *et al.*, 1998), and this is consistent with our observation that the MIC of the mutant for bacitracin is higher in blood-containing media, although it is unaffected by serum concentration. As the higher MIC of wild-type *Strep. pneumoniae* did not vary, it appears that at lower bacitracin concentrations some medium component is

binding to and sequestering the antibiotic. It is, however, clear that the *bacA* mutation considerably increases *Strep. pneumoniae* susceptibility to bacitracin, suggesting that it directly and specifically affects the role of UPP in this organism. We hypothesize that in the *bacA* mutant, UP is produced solely by dephosphorylation of UPP. Hence, when this intermediate is sequestered by bacitracin, UP supplies are rapidly recycled out of circulation and cell wall biosynthesis ceases altogether.

The *bacA* mutants and their parent strains showed no detectable differences in morphology, growth parameters or (in the case of *Staph. aureus*) sensitivity to osmotic stress which might indicate a deficiency in cell wall synthesis in the absence of bacitracin. However, the *Strep. pneumoniae bacA* mutant was severely attenuated in a mouse RTI infection model. This suggests that, although the *bacA* mutant cells were healthy *in vitro*, the absence of undecaprenol kinase causes metabolic changes sufficient to compromise infectivity. The *Staph. aureus* WCUH29 *bacA* mutant also showed reduced infectivity in a murine infection model and increased susceptibility to bacitracin, but the effects of the mutation were surprisingly slight in comparison to *Strep. pneumoniae*. Although we have shown that the undecaprenol kinase protein described by Sandermann & Strominger (1971) is very likely to be the *bacA* gene product, the possibility that *Staph. aureus* contains a second, uncharacterized, undecaprenol kinase activity cannot be ruled out. Previous studies have shown that in some organisms the size of the undecaprenol pool varies according to growth phase (Thorne, 1973), so it is also possible that the size of the pool and the importance of undecaprenol as an alternative source of UP also vary between organisms. Alternatively, environmental stresses specific to the *Strep. pneumoniae* infection model may exacerbate the deficiencies of the *Strep. pneumoniae bacA* mutant in establishing and/or maintaining infection.

Novel agents targeting *Strep. pneumoniae* are of particular interest, as this organism is the most common cause of community-acquired pneumonia requiring hospitalization, with high morbidity and mortality rates (Marrie, 1999). It also causes meningitis, acute otitis media and acute exacerbations of chronic bronchitis, and increasing rates of resistance to common antibiotics including penicillins and macrolides are reported. Our data suggest that an inhibitor of BacA would not only reduce *Strep. pneumoniae* virulence in its own right, but would also have a synergistic effect on bacitracin activity which is potentially sufficient to reduce the effective bacitracin dose to below toxic levels. If so, such an antibiotic combination could theoretically be used without toxic effect in systemic applications for which bacitracin monotherapy is unsafe.

Finally, in this work we have followed nomenclature used by Cain *et al.* (1993). However, we note that the gene name *bacA* is not unique, as it is also used for bacitracin synthetase 1 of *Bacillus licheniformis*, and for a transport protein involved in bacteroid synthesis in *Rhizobium* spp. We therefore propose that consideration is given to an alternative designation (for example *bsdA*, for bacitracin susceptibility determinant) for this gene.

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