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SHORT ARTICLE

Increases in the mutation frequency at which fusidic acid-resistant *Staphylococcus aureus* arise with salicylate

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Salicylate was shown to increase the frequency at which a fusidic acid-susceptible strain of *Staphylococcus aureus* underwent mutation to become fusidic acid-resistant. These fusidic acid-resistant mutants had alterations in spectinomycin and kanamycin resistance levels indicative of mutations in *fus*A, the gene that encodes elongation factor-G, the target of fusidic acid.

Introduction

Previous studies showed that growth of *Staphylococcus aureus* in the presence of salicylate increased phenotypic resistance to fusidic acid and fluoroquinolones [1, 2]. Fluoroquinolone-resistant mutants of *S. aureus* were also shown to arise at a greater frequency in the presence of salicylate [2]. As an extension to these studies, the effect of salicylate on the frequency of mutation to fusidic acid resistance in a fusidic acid-susceptible *S. aureus* strain was determined.

Materials and methods

Strains and population analyses

The well characterised *S. aureus* strain, BB255, was used in this study [3]. Fusidic acid-resistance population analyses were performed as described previously [1] on Luria Broth Agar (LBA; Difco) plates containing fusidic acid, with and without the addition of 2 mM salicylate, ibuprofen, benzoate, acetaminophen, saligenin or acetate. Suspected fusidic acid-resistant colonies were subcultured from these plates and grown three times in drug-free Luria Broth (LB) overnight at 37°C and then stored in LB with glycerol 20% v/v at −20°C.

MIC determinations

Fusidic acid MIC determinations were performed by the agar dilution technique on Mueller-Hinton Agar (Oxoid) according to NCCLS guidelines. The gradient-plate technique was used to determine kanamycin (0–4 mg/L gradient) and spectinomycin (0–150 mg/L gradient) resistance levels as described previously [4].

Chemicals

Stock solutions of sodium salicylate (1 M), sodium acetate (1 M), sodium benzoate (0.5 M) (BDH Laboratory Supplies, Poole) and sodium ibuprofen (0.5 M) (Sigma) were made up in water. Acetaminophen (0.5 M), acetylsalicylic acid (0.5 M) (ICN Pharmaceuticals, Costa Mesa, CA, USA) and saligenin (0.5 M) (Sigma) stocks were made up in ethanol. The pH of these solutions was adjusted to 7 and, when required, they were filter-sterilised. All stock solutions were stored in dark containers at 4°C. Sodium fusidate (Leo Pharmaceuticals, Ballerup, Denmark), kanamycin and spectinomycin (Sigma) were dissolved in water, filter-sterilised and stored at −20°C.

Results and discussion

The addition of salicylate, ibuprofen or benzoate to media used in fusidic acid-resistance population analyses increased the number of cells surviving at otherwise inhibitory concentrations of fusidic acid (Fig. 1). The effect of ibuprofen on cell survival in the presence of fusidic acid was similar to that of salicylate, whereas the enhancement of cell survival by benzoate was less than with these compounds (Fig. 1). Acetaminophen, saligenin (the alcohol of salicylate) and acetate had no effect on fusidic acid-resistance population analyses in this study (data not shown).

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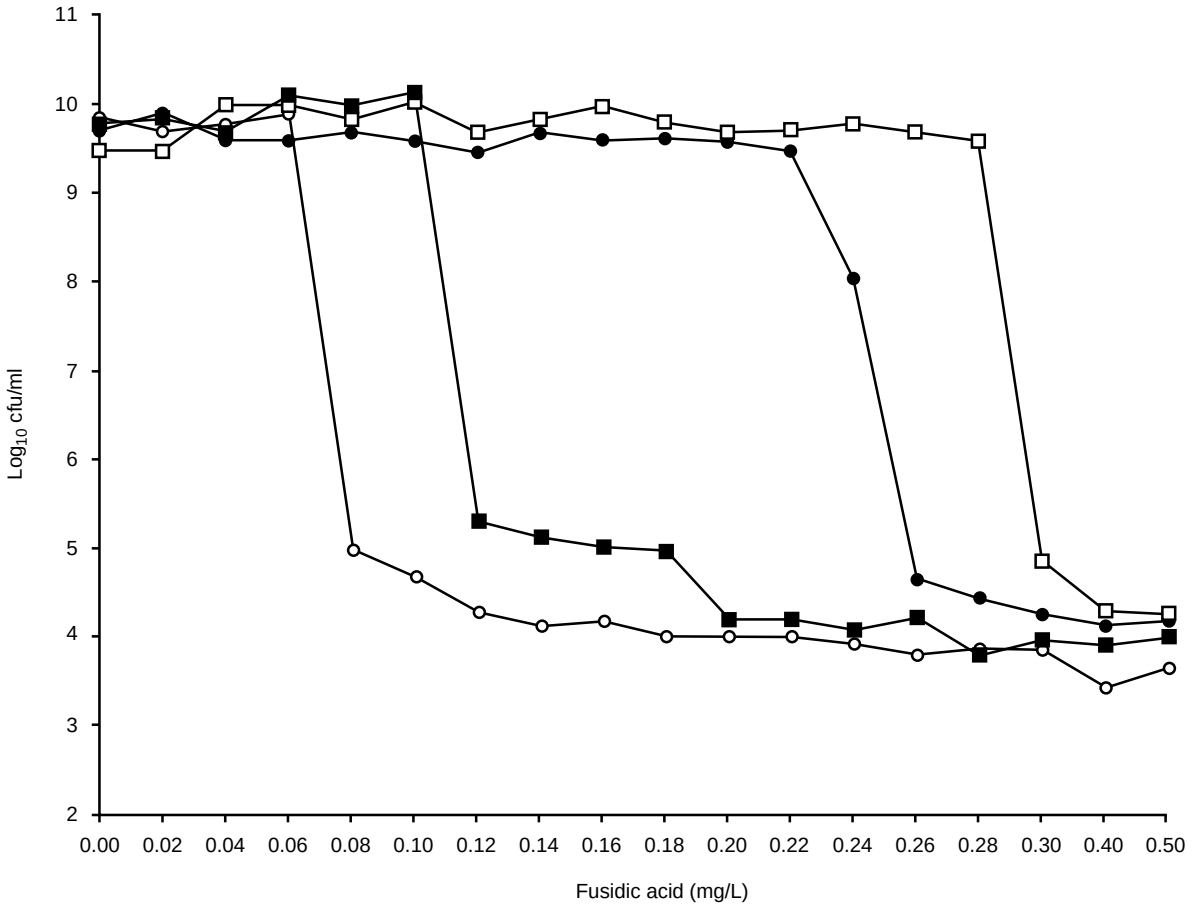

Fig. 1. Population analysis of strain BB255 in the presence of fusidic acid alone (○) or with 2 mM salicylate (●), ibuprofen (□) or benzoate (■).

These data correlate with previous studies that demonstrated that compounds with an aromatic ring and a carboxylic acid functional group induce intrinsic fusidic acid resistance in *S. aureus* [1].

Individual colonies of strain BB255 surviving on plates containing fusidic acid 0.24 and 0.26 mg/L, with and without 2 mM salicylate, were isolated as described above and their fusidic acid MICs were determined (Table 1). Colonies surviving on fusidic acid 0.24 mg/L and 2 mM salicylate had not become fusidic acid resistant by mutation (Table 1). It has been shown previously that salicylate can induce a phenotypic multiple antibiotic resistance mechanism in *S. aureus* [1, 2]. All colonies selected from plates containing only fusidic acid (0.24 and 0.26 mg/L) and fusidic acid 0.26 mg/L and 2 mM salicylate had undergone mutation to become fusidic acid-resistant (Table 1). In the presence of salicylate and fusidic acid 0.26 mg/L, the mutation frequency to become fusidic acid-resistant was 10-fold greater than with fusidic acid 0.26 mg/L alone (Table 2). This demonstrates that salicylate can increase the mutation frequency of a susceptible strain of *S. aureus* to become resistant to fusidic acid.

Fusidic acid inhibits the translocation step of protein synthesis by interfering with the action of elongation factor-G (EF-G) [5]. Kanamycin and spectinomycin also interfere with the translocation step of protein synthesis [6–8]. Mutations leading to fusidic acid resistance in bacteria occur in *fus*A, the gene encoding the target of fusidic acid, EF-G [4, 9, 10]. *fus*A mutations leading to fusidic acid resistance also alter resistance to kanamycin and spectinomycin in bacteria [4, 10]. Kanamycin and spectinomycin gradient-plate MICs were determined for fusidic acid-resistant BB255 isolates from plates containing fusidic acid 0.26 mg/L with and without 2 mM salicylate. Compared with strain BB255, all these isolates had increased kanamycin and reduced spectinomycin gradient-plate MICs, with the exception of isolate CP70, which demonstrated increased spectinomycin resistance (Table 3). Therefore, it is probable that these isolates all have mutations in *fus*A, which leads to increased fusidic acid resistance.

This report and a previous study [2] demonstrate that the addition of salicylate can increase the mutation frequency of *S. aureus* at unrelated chromosomal loci *in vitro*, dependent on the antibiotic selection. How salicylate induces an increased mutation frequency to fusidic acid or fluoroquinolone resistance in *S. aureus* is unclear at present. Incorporation of salicylate into the Ames test under four different metabolic conditions failed to increase the revertant colony counts, indicating that salicylate has no mutagenic potential [11].

Table 1. Fusidic acid MICs of BB255 isolates from fusidic acid-resistance population analysis plates

No salicylate			2 mM salicylate		
Isolate no.	Fusidic acid concentration (mg/L)	MIC (mg/L)	Isolate no.	Fusidic acid concentration (mg/L)	MIC (mg/L)
BB255	0	0.25	CP73	0.24	0.25
CP63	0.24	16	CP74	0.24	0.25
CP64	0.24	16	CP75	0.24	0.25
CP65	0.24	16	CP76	0.24	0.25
CP66	0.24	16	CP77	0.24	0.25
CP67	0.24	8	CP78	0.26	4
CP68	0.24	8	CP79	0.26	4
CP69	0.26	16	CP80	0.26	4
CP70	0.26	16	CP81	0.26	8
CP71	0.26	16	CP82	0.26	8
CP72	0.26	8	CP83	0.26	4

Table 2. BB255 fusidic acid resistance mutation frequencies

Fusidic acid concentration in plate (mg/L)	Addition of 2 mM salicylate	Mutation frequency to Fa^R
0.24	–	1.29×10^{-6}
0.24	+	–
0.26	–	1.00×10^{-6}
0.26	+	1.00×10^{-5}

Table 3. Kanamycin and spectinomycin gradient plate MICs

Isolate no.	Kanamycin MIC (mg/L)	Spectinomycin MIC (mg/L)
BB255	1.2	77.5
CP69*	>4	15
CP70	>4	106.25
CP71	>4	25
CP72	>4	26.25
CP78†	>4	70
CP79	1.73	57.5
CP80	2.47	63.75
CP81	>4	67.5
CP82	>4	48.75
CP83	>4	61.25

*CP69–CP72 isolated from fusidic acid 0.26 mg/L.
†CP78–CP83 isolated from fusidic acid 0.26 mg/L and 2 mM salicylate.

As growth in the presence of salicylate has a number of different effects on bacterial phenotypes [1, 2, 12–17], it is probable that salicylate-altered gene expression plays a role in altering mutation frequencies to antibiotic resistance during antibiotic resistance selection. Thus, research to determine the effects of salicylate and related compounds on the efficacy of antistaphylococcal therapies *in vivo* is warranted.

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