Osmoprotective properties and accumulation of betaine analogues by Staphylococcus aureus

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Abstract

Betaines were evaluated as potential antistaphylococcal agents for urinary tract infections. Staphylococcus aureus accumulated all tested betaines except trigonelline. S. aureus transport systems were less sensitive to carbon chain length than those of Escherichia coli. Betaines were accumulated in the absence of osmotic stress, and 10-fold more in hyperosmotic medium. Most betaines increased the osmotolerance of S. aureus in defined minimal medium. Unlike E. coli, S. aureus did not significantly accumulate a second betaine in the presence of glycine betaine. Betaines are less likely to be useful in treating staphylococcal than E. coli urinary infections. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: Betaine; Staphylococcus aureus; Osmoprotection; Osmolyte; Antimicrobial agent

1. Introduction

Urinary tract infection is a common cause of consultations in general practice and hospital admissions, and, in consequence, of antibiotic prescription (not always appropriate). The current rise in antibiotic resistance now includes vancomycin resistance in staphylococci [1]. Although recognition of Staphylococcus saprophyticus as a urinary tract pathogen was slow in coming, it is now known to be the second most common cause (after Escherichia coli) of urinary tract infections [2].

The ability of bacteria to accumulate betaines as osmoprotectants might be exploited to produce an antibacterial betaine [3–5]. Both Gram-negative and Gram-positive bacteria will accumulate betaines under hyperosmotic conditions, but the role of the betaines differs for different species. For Gram-negative bacteria, the betaines act as osmoprotectants at osmolalities which occur in the urinary tract [6]. S. aureus, however, is among the most osmotolerant of the non-halophilic eubacteria. It grows in NaCl concentrations up to 3.5 mol l⁻¹, and also grows well at low osmolalities, suggesting that the organism has efficient means of regulating cytoplasmic osmolality.

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However, in defined medium, growth is inhibited by osmotic stress, and glycine betaine is the most effective osmoprotectant agent, especially at high levels of osmolality [7]. Urinary isolates of *S. aureus* and *S. saprophyticus* accumulate glycine betaine with and without osmotic stress [8]. Proline betaine, which accumulates through the proline transport system, is another potent osmoprotectant for *S. aureus* [9].

In order to see whether the characteristics of accumulation by *S. aureus* are comparable to those of *E. coli*, the ability of *S. aureus* to accumulate a range of naturally occurring and synthetic betaines singly and in competition with glycine betaine was investi-
gated using a laboratory strain of *S. aureus*. The osmoprotective activity of the selected betaines in defined medium was also assessed.

2. Materials and methods

The test strain used was *S. aureus* ATCC 25923.

2.1. Betaines

The betaines used were (Fig. 1): (1) homologues of glycine betaine: glycine betaine, *n* = 1; propionobetaine, *n* = 2; butyrobetaine ((3-carboxypropyl) trimethylammonium chloride), *n* = 3; caprobetaine, *n* = 5; (2) *K*-substituted betaines: L-alanine betaine, L-proline betaine, L-serine betaine, L-valine betaine; (3) betaines with different cationic groups: trigonelline, pyridinium betaine, dimethylsulfoniopropionate (DMSP), *N*-phenylglycine betaine, dimethylthetin, thiolanium betaine, triethylglycine betaine.

Betaines not commercially available (Sigma Chemical Corp., St. Louis, MO) were synthesised as summarised by Randall et al [10].

2.2. Culture conditions

The defined growth medium of Pattee and Neveln [11] without nucleic acids and agar was used throughout. Proline levels were altered to 10 μmol l⁻¹, which is insufficient to serve in an osmoregulatory role [9]. Osmolality of this medium was 318 mosmol kg⁻¹, measured by freezing point depression (Advanced Digimatic Osmometer model 3D2). For assessing the relationship between bacterial growth and osmolality in the presence of betaines, NaCl was added up to 2 mol l⁻¹ (4170 mosmol kg⁻¹), and the pH adjusted to 7.0. Optical density at 580 nm after 12 h incubation was used as a measure of growth.

Growth curves for *S. aureus* in media with and without 0.6 mol l⁻¹ NaCl (osmolality 1361 mosmol kg⁻¹) and with no added betaine, using OD measurements (580 nm) and viable counts (serial dilution), showed stationary phase growth in both media at 16 h.

For betaine accumulation, an inoculum from overnight culture in the defined medium was transferred to fresh medium with and without 0.6 mol l⁻¹ NaCl. Betaines were added to give a final concentration of 500 μmol l⁻¹. Intracellular glycine betaine was measured at eight time points between four and 24 h incubation at 37°C in a shaking waterbath. For assessment of accumulation of a range of different betaines, cultures were incubated 15 h. To assess accumulation of a second betaine at five and 16 h, equimolar amounts of glycine betaine and a second betaine were added (500 μmol l⁻¹ final concentration of each).

To determine whether the known proline port was involved in betaine accumulation, betaine accumulation in the presence of excess proline was measured. Defined minimal media at 318 and 1366 mosmol kg⁻¹ were made, with 20 μmol l⁻¹ proline and 2000 μmol l⁻¹ proline, each medium. Betaines were added singly, and accumulation by *S. aureus* measured after 16 h incubation.

![Fig. 2. Variation in intracellular glycine betaine concentration with time of incubation in medium containing betaine. Circles: betaine accumulation from medium at 318 mosmol kg⁻¹; triangles: betaine accumulation from medium at 1360 mosmol kg⁻¹.](image-url)
2.3. Analytical methods

Betaines were measured in bacterial cell extracts, after washing in water or equiosmolar NaCl to remove extracellular betaine, by extraction into acetonitrile containing 10% methanol, sonication (10 min) and centrifugation. The supernatant was removed for betaine assay using HPLC methods [10,12]. The elution solvent was 75% buffered isopropl alcohol. Valine betaine was separated on an alumina column.

Osmoprotection was assessed using calculations previously described [12] with the empirical function adjusted to give 50% growth.

Experiments were carried out in quadruplicate except for accumulation over time (duplicates).

3. Results

All betaines except N-phenylglycine betaine increased the maximum osmolality which supported growth (Table 1). The increase was significant for glycine betaine, propionobetaine, L-proline betaine, dimethylthetin and thiolanium betaine \((P < 0.05)\). The highest levels of intracellular betaine were found in the samples taken at 4 h incubation for both osmolalities tested (Fig. 2). Beyond 4 h, intracellular betaine dropped and reached a plateau by 10–12 h.

After 16 h incubation all betaines in the homologous series and all the \(\alpha\)-substituted betaines tested were accumulated, with the accumulation in the 1361 mosmol kg\(^{-1}\) medium being approximately one log higher (Table 1). Of the modified cationic betaines, trigonelline and pyridinium betaine were minimally accumulated.

When betaines were added paired with glycine betaine, the second betaine was very poorly accumulated (Table 2). Only L-proline betaine and dimethylthetin were consistently accumulated to more than 1% of the glycine betaine at both osmolalities. There was little difference between the proportions of the second betaine at 5 and 16 h.

Intracellular betaine levels were not affected by varying the proline concentration in the media.

Table 1
Osmoprotective activity and intracellular accumulation of betaines by *Staphylococcus aureus* grown in defined minimal medium

<table>
<thead>
<tr>
<th>Betaine</th>
<th>Half-growth osmolality(^a) (mosmol kg(^{-1}))</th>
<th>Intracellular betaine (amol cell(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>No betaine</td>
<td>2199 ± 202</td>
<td>–</td>
</tr>
<tr>
<td><strong>Homologues of glycine betaine:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine betaine</td>
<td>3969 ± 128*</td>
<td>90</td>
</tr>
<tr>
<td>Propionobetaine</td>
<td>3407 ± 147*</td>
<td>41</td>
</tr>
<tr>
<td>Butyrobetaine</td>
<td>2817 ± 173</td>
<td>35</td>
</tr>
<tr>
<td>Caprobetaine</td>
<td>2648 ± 215</td>
<td>16</td>
</tr>
<tr>
<td><strong>(\alpha)-Substituted betaines:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Proline betaine</td>
<td>3256 ± 145*</td>
<td>65</td>
</tr>
<tr>
<td>L-Alanine betaine</td>
<td>2402 ± 444</td>
<td>16</td>
</tr>
<tr>
<td>L-Serine betaine</td>
<td>2644 ± 164</td>
<td>16</td>
</tr>
<tr>
<td>L-Valine betaine</td>
<td>3002 ± 176</td>
<td>6</td>
</tr>
<tr>
<td><strong>Other betaines:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trigonelline</td>
<td>2619 ± 271</td>
<td>0.4</td>
</tr>
<tr>
<td>Pyridinium betaine</td>
<td>2820 ± 154</td>
<td>6</td>
</tr>
<tr>
<td>N-Phenylglycine betaine</td>
<td>2011 ± 330</td>
<td>9</td>
</tr>
<tr>
<td>Triethylglycine betaine</td>
<td>3540 ± 167</td>
<td>38</td>
</tr>
<tr>
<td>Dimethylthetin</td>
<td>3421 ± 137*</td>
<td>41</td>
</tr>
<tr>
<td>Dimethylsulfiniopropionate</td>
<td>2974 ± 224</td>
<td>11</td>
</tr>
<tr>
<td>Thiolanium betaine</td>
<td>3554 ± 165*</td>
<td>nd</td>
</tr>
</tbody>
</table>

\(^a\)Osmolality at which growth of *S. aureus* was 50% of growth in defined minimal medium without betaine.

*Significant salt protector: \(P < 0.05\).
4. Discussion

Although *S. aureus* ATCC 25923 will grow in the presence of salt over the range of osmolalities used in these experiments, most betaines tested increased its osmotolerance. Effective betaines were accumulated in the presence or absence of osmotic stress. This accords with the presence of both high and low affinity glycine betaine systems with the low affinity system being activated by osmotic stress [13,14]. Our experience was that accumulation of all betaines increased by a logarithmic order under osmotic stress (1361 mosmol kg\(^{-1}\)). *E. coli*, on the other hand, does not accumulate betaines from defined minimal medium without added NaCl [6,8].

Although there are differences between *S. aureus* and *E. coli* in the betaines which are accumulated, *S. aureus*, like *E. coli* [6,15], has non-specific betaine transporters. The low-affinity system has been said to be inhibited by proline [14], but we were unable to demonstrate changes in accumulation levels when excessive amounts of proline were supplied in the medium at either osmolality. None of the tested betaines could be demonstrated to be accumulated at one osmolality and not the other.

Betaine accumulation by *E. coli* depends on betaine structure. The separation between charged molecules is important, accumulation declining as the number of carbon atoms increased beyond one [6]. *S. aureus* differs from *E. coli* in that the transport systems are less sensitive to chain length and will accumulate caprobetaine which has a 5-carbon separation between the charges, while *E. coli* does not. *E. coli* accumulates trigonelline (although it is ineffective as an osmoprotectant), while *S. aureus* does not [6,15]. Both species accumulate the \(\alpha\)-substituted betaines well.

With *E. coli*, the \(\alpha\)-substituted betaines, the homologous betaines with \(n \leq 2\) and trigonelline were accumulated almost as well as glycine betaine by 16 h when supplied together with glycine betaine [15]. Unlike *E. coli*, *S. aureus* did not accumulate any other betaine well in the presence of glycine betaine under the experimental conditions used. At concentrations of 500 \(\mu\)mol l\(^{-1}\) of each betaine, there would still be extracellular glycine betaine available for preferential uptake. With lower concentrations such as urinary glycine betaine levels [12,16], it may be that the second betaine would be accumulated once most of the glycine betaine had been taken up.

Apart from the \(\alpha\)-substituted betaines, it would appear that any betaine likely to be accumulated by *S. aureus* is also likely to be accumulated by Madin Darby canine kidney cells [17]. This, together
with the low accumulation of a second betaine, gives fewer openings for developing an antibacterial betaine to be used for treatment of staphylococcal urinary tract infections than for developing an anti-\textit{E. coli} betaine. The same agent would be unlikely to inhibit both \textit{E. coli} and \textit{S. aureus}. However, it may be possible to exploit the ability of \textit{S. aureus} to accumulate a wide variety of betaines in the absence of glycine betaine in the treatment of infection at non urinary tract sites. We calculate that the concentration of betaine inside the staphylococcal cell in our experimental system is likely to be in the order of $30$–$200$ mmol l$^{-1}$ at $318$ mosmol kg$^{-1}$ and $500$–$2000$ mmol l$^{-1}$ at $1361$ mosmol kg$^{-1}$. (Most antibiotics are active in the order of $10^{-3}$ to $10^{-5}$ molar.) There may be potential for betaines in treatment of staphylococcal skin infections.

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**References**


