The Characteristics of Peptide Uptake in *Streptococcus faecalis*: Studies on the Transport of Natural Peptides and Antibacterial Phosphonopeptides

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Transport of natural peptides and antibacterial phosphonopeptide analogues was studied in *Streptococcus faecalis* ATCC 9790. Competition studies, and the isolation of peptide-transport deficient mutants, indicate the presence of two peptide permeases. One is a high-rate system used by dipeptides, and to a lesser extent tripeptides; the other is a low-rate oligopeptide system. Following uptake, peptides are cleaved and their amino acid residues may undergo rapid exodus. Different strains of *S. faecalis* differ in their rates of peptide transport.

INTRODUCTION

There is an extensive literature on peptide transport in bacteria, and this has been the subject of recent reviews (e.g. Payne & Gilvarg, 1978; Payne, 1980). However, much of the detailed work has used *Escherichia coli* as the test organism, and information on *Streptococcus faecalis* is sparse. Early work (Kihara *et al.*, 1952, 1961) showed that peptides can serve as better amino acid sources for *S. faecalis* than the free residues, and [¹⁴C]Gly-Gly uptake was later demonstrated to be uninhibited by free alanine or glycine (Brock & Woolley, 1964), implying a distinction between amino acid and peptide transport. However, the uptake system(s) for peptides have not been characterized.

In the present work we have studied the uptake of a variety of natural peptides and some representatives of the antibacterial phosphonopeptides (Atherton *et al.*, 1979). Peptide uptake was shown to be independent of peptidase action, and strain differences in the specificity of uptake were demonstrated. Exodus of amino acid residues after uptake and cleavage of peptide was shown. By a combination of competition studies and the isolation of peptide transport-deficient mutants, one strain of *S. faecalis* was shown to possess two peptide permeases of different specificity.

A preliminary account of part of this work has appeared (Nisbet & Payne, 1980).

METHODS

Organisms. *Streptococcus faecalis* (*faecium*) strain ATCC 9790 was obtained from Professor F. M. Harold, National Jewish Hospital, Denver, Colorado, U.S.A.; *S. faecalis* strains OG-FS5 and NCIB 6459 were obtained from Dr P. S. Ringrose, Roche Products Ltd, the former having originally been isolated from a clinical source by Professor F. W. O'Grady, Department of Microbiology, Nottingham University, U.K.

Media and growth conditions. In initial studies, organisms were maintained on SF35 medium (Andrew, 1973) containing agar (1.5%, w/v), and grown in liquid culture in the same medium without agar. In the majority of studies reported here, organisms were maintained on RST2 medium (Atherton *et al.*, 1979) with a vitamin supplement (Allen *et al.*, 1979) containing agar (1%, w/v), and were grown in liquid culture in the same medium without agar. Unless stated in Results, the use of RST2 medium can be assumed.

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Assay of sensitivity to antibacterial compounds. Organisms were grown overnight in liquid culture at 37 °C. Portions of the culture (0.1 ml, approx. 10^8 organisms) were spread on agar plates, and the surface of the plate was allowed to dry for about 20 min at room temperature. A portion (10 µl) of a solution of the antibacterial compound to be tested was then pipetted on to the centre of the plate, which was incubated overnight at 37 °C. Growth inhibition was measured by the size of the zone in which no growth occurred.

Assay of peptide transport. Uptake of non radioactively labelled peptides was assayed by using a modification of a method in which fluorescamine (Nisbet & Payne, 1979) is used to determine the rate of disappearance of substrate from the medium. In the present studies the procedure was automated, a multichannel peristaltic pump being used both to sample the incubation mixture and to introduce the correct proportion of buffer and of fluorogenic reagent (Payne & Nisbet, 1981). Briefly, organisms were grown to a cell density of 0.1–0.3 mg dry wt ml⁻¹, harvested by membrane filtration (Oxoid, 0.45 µm pore size, 25 mm diameter) and washed and resuspended to a density of 0.1–0.3 mg dry wt ml⁻¹ in K₂HPO₄/KH₂PO₄ buffer (pH 6.9, 50 mM with respect to phosphate) containing glucose (0.2% w/v). After pre-incubation (10 min, 37 °C), peptide (0.1 mM) was added and sampling initiated. The rate of uptake of peptide was routinely measured during the first 5 min of incubation.

When radioactively labelled substrates were used, organisms were grown as above and harvested by centrifugation, being washed and resuspended in phosphate buffer as above. After preincubation (10 min, 37 °C), labelled peptide (0.1 mM) was added; samples (500 µl) were removed periodically, collected on glass-fibre filters (Whatman GF/C), and washed with saline (2 × 5 ml, 0.9% w/v). The filters were placed in scintillation vials and dried at 80 °C for 45 min. Scintillation fluid (BBOT in toluene, 5 ml) was added to each vial, and the vials were counted for ¹⁴C using a Packard model 3320 Tri-Carb scintillation spectrophotometer. Efficiency of counting was approximately 70%. With labelled peptides it can be difficult to obtain true rates of uptake because of exodus and metabolism (Payne & Nisbet, 1980), but this procedure has been shown to give true rates provided that measurements are restricted to about the first 30 s of incubation. Thus, peptides containing [¹⁴C]Ala were sampled every 8 s up to 40 s, while those containing [¹⁴C]Ala(P) (see below) were sampled every 2 min for 8 min, as this residue does not undergo exodus after intracellular cleavage (Nisbet, 1980; Atherton *et al.*, 1979).

Peptide uptake and amino acid exodus were also monitored by chromatography of dansyl derivatives of incubation media and cell extracts (Payne & Bell, 1979). To prepare cell extracts bacteria were collected on membrane filters (Oxoid, 0.45 µm pore size, 25 mm diameter) and washed with phosphate buffer (as above), and the filters were placed in boiling water (1 ml per ml incubation) containing toluene (5%, v/v) for 15 min.

Materials. Roche Sensitivity Test Medium Type 2 (RST2) was a gift from Dr M. J. Hall, Roche Products Ltd, Welwyn Garden City, Herts., U.K. The alanine analogue [¹⁴C]aminoethylphosphonic acid ([¹⁴C]Ala(P)), the peptide analogues, Ala-[¹⁴C]Ala(P), Ala-Ala-[¹⁴C]Ala(P), Ala-Ala-Ala-[¹⁴C]Ala(P), the peptides Ala-[¹⁴C]Ala, Ala-Ala-[¹⁴C]Ala, Ala-Ala-Ala-[¹⁴C]Ala, and fluorescamine (Fluram) were gifts from Dr W. J. Lloyd and Dr P. S. Ringrose, Roche Products Ltd. The alanine analogue L-aminoxypionic acid (AXOL), and the peptide analogues Ala-L-aminoxypionic acid (AX1) and Pro-Ala-L-aminoxypionic acid (AX5) were gifts from Dr J. S. Morley, ICI Pharmaceuticals, Alderley Park, Cheshire, U.K. All other peptides and amino acids were purchased from Sigma or from Uniscience Ltd, Cambridge, U.K. Dansyl chloride and polyamide sheets for thin-layer chromatography (15 × 15 cm) were from BDH and 2,5-bis(5-*tert*-butylbenzoxazol-2-yl) thiophene (BBOT) was from Fisons Scientific Apparatus. All other reagents were of analytical grade.

RESULTS

Isolation of peptide-transport-deficient mutants. Strain ATCC 9790 was plated out in the presence of the toxic tripeptide analogue AX5 (10 µl of 20 mM solution). After incubation, isolated colonies were present in the growth inhibition zone. One of these was removed, grown overnight in liquid medium, serially diluted, and plated again in the presence of AX5. After growth, a single colony was taken, grown up, and designated strain PA 6102.

Strain PA 6102 was plated out in the presence of the toxic dipeptide analogue AX1 (10 µl of a 20 mM solution). Strain PA 610201 was isolated from a colony growing in the inhibition zone, by using a similar process to that above. The resistance patterns of strains ATCC 9790, PA 6102, and PA 610201 are summarized in Table 1.

Intact accumulation of peptide. The demonstration of intact peptide accumulation is usually difficult, as most substrates of peptide permeases are rapidly cleaved by intracellular peptidases. However, glycylsarcosine, whilst retaining adequate affinity for uptake, is resistant to peptidase action, properties that have allowed its intact accumulation to be demonstrated in *E. coli* (Payne & Bell, 1977, 1979) and *Saccharomyces cerevisiae* (Nisbet & Payne, 1979). Similarly, intact accumulation was shown here for *S. faecalis* (Table 2) by

Table 1. *Characteristics of strain ATCC 9790 and its derivatives*

About 10^8 exponential-phase bacteria were spread on an SF35 agar plate: peptide inhibitor (10 μ l, 20 mM) was spotted in the centre of the plate, which was then incubated for 18 h at 37 °C.

Strain	Isolated as resistant to	Derived from	Response to antibacterial peptides			
			AX1		AX5	
			Diam. of inhibition zone (mm)	No. of isolated colonies	Diam. of inhibition zone (mm)	No. of isolated colonies
ATCC 9790	—	—	30	0	25	>100
PA 6102	Pro-Ala-aminoxypionic acid (AX5)	ATCC 9790	40	20	0	—
PA 610201	Ala-aminoxypionic acid (AX1)	PA 6102	0	—	0	—

Table 2. *Accumulation of glycylsarcosine by S. faecalis ATCC 9790*

Organisms were suspended in SF35 medium to a density of 0.15 mg dry wt ml⁻¹. After pre-incubation (10 min, 37 °C), glycylsarcosine (1 mM) and a potential competitor (1 mM) were added, and the mixture was incubated for 30 min. Cell extracts were examined for intact glycylsarcosine accumulation by using dansylation. Intracellular concentrations were calculated using an intracellular volume of 1.75 μ l (mg dry wt)⁻¹ (Bakker & Harold, 1980).

Competitor	Intracellular concn of glycylsarcosine (mM)
None	115
1 mM-Ala-Ala	60
1 mM-Leu-Leu	75
1 mM-Ala-Ala-Ala	100
1 mM-Ala-Ala-Ala-Ala	115

dansylation of cell extracts. Glycylsarcosine uptake was subject to inhibition by other transportable peptides, for example, Ala-Ala (Fig. 1, E–F), Leu-Leu, Ala-Ala-Ala (Table 2).

Amino acid exodus during peptide uptake. For peptides that are rapidly transported and cleaved intracellularly, large internal concentrations of the constituent amino acids can result, although these amino acids may undergo considerable exodus, this being demonstrable by dansylation of samples of the incubation medium (e.g. exodus of Ala during Ala-Ala uptake, Fig. 1, A–C). This exodus can invalidate measurements of uptake using labelled peptides (Payne & Nisbet, 1980). It is stimulated by extracellular amino acids. For example, free alanine, although not affecting the initial rate of Ala-Ala uptake by strain ATCC 9790, lowered the amount of label accumulated over a longer time course by increasing [¹⁴C]Ala exodus (Fig. 2). This implies that the exodus occurs via the general amino acid permease (Brock & Moo-Penn, 1962; Holden & Bunch, 1973), for which exchange of intracellular and extracellular amino acids has been shown (Asghar *et al.*, 1973).

Peptide uptake in strain ATCC 9790 and derivative strains. Strain ATCC 9790 transported a range of natural dipeptides and tripeptides, but uptake of Ala-Ala-Ala-Ala was undetectable (Table 3). Phosphonopeptides (Atherton *et al.*, 1979), which contain the antibacterial moiety aminoethylphosphonic acid [Ala(P)], were also transported, although at low rates (Table 3). In contrast, uptake of free Ala(P) was undetectable when assayed under similar conditions.

Fig. 1. Chromatograms of dansylated peptides and amino acids from incubation media and cell extracts of *S. faecalis* during peptide uptake. A to C. Samples of medium during incubation of strain ATCC 9790 ($0.18 \text{ mg dry wt ml}^{-1}$) with Ala-Ala (0.1 mM). A, 0 min; B, 5 min; C, 10 min. D, Key to derivatives: a, Ala-Ala; b, Ala; c, Gly; d, Pro; f, Orn (internal standard); g, dansyl hydroxide; h, dansyl ammonia; i, origin.

E to G. Cell extracts of strain ATCC 9790 ($0.18 \text{ mg dry wt ml}^{-1}$) after 20 min incubation with: E, glycylsarcosine (1 mM); F, glycylsarcosine (1 mM) + Ala-Ala (1 mM); G, no peptide. H. Key to derivatives (scale enlarged for clarity): a, Ala; b, Gly; c, Glu; d, Asp; e, Pro; f, Val; g, Leu; h, Orn; i, Lys; j, Thr; k, Ser; l, Arg; m, glycylsarcosine; n, Ala-Ala; p, dansyl ammonia; q, diaminopimelic acid (internal standard); r, dansyl hydroxide; s, origin.

Fig. 2. Effect of Ala on Ala- $[^{14}\text{C}]$ Ala uptake by *S. faecalis*. Strain ATCC 9790 was suspended in potassium phosphate buffer (pH 6.9, 50 mM) plus glucose (0.2% , w/v) to a density of $0.15 \text{ mg dry wt ml}^{-1}$. Uptake of label from Ala- $[^{14}\text{C}]$ Ala (0.1 mM) was followed in the absence (O) and presence (●) of unlabelled Ala (1 mM).

Compared with the parent strain, uptake of natural peptides and of Ala-Ala(P) was apparently unaltered in the mutant PA 6102 (resistant to the toxic tripeptide AX5), whereas uptake of Ala-Ala-Ala(P) and Ala-Ala-Ala-Ala(P) was undetectable (Table 3). These results are consistent with the existence of an oligopeptide transport system that handles Ala-Ala-Ala(P), Ala-Ala-Ala-Ala(P) and AX5, and which is lost in the mutant PA 6102.

Mutant PA 610201, which is also resistant to the toxic dipeptide AX1, was deficient in the uptake of all peptides tested (Table 3), an observation consistent with the existence of a

Table 3. Uptake of peptides by strain ATCC 9790 and derivative strains

Organisms were suspended in potassium phosphate buffer (pH 6.9, 50 mM) plus glucose (0.2%, w/v) to a density of 0.1–0.3 mg dry wt ml⁻¹. Uptake of peptide was assayed by the standard fluorescamine (F) or radiotracer (R) method. Uptake rates are the range of from one to five determinations.

Peptide (0.1 mM)	Assay	Rate of uptake [nmol min ⁻¹ (mg dry wt) ⁻¹]		
		ATCC 9790	PA 6102	PA 610201
Ala-Ala	F	92–103	93–106	ND
Ala-Ala	R	98–100	98	1
Lys-Lys	F	13–20	18	ND
Leu-Leu	F	58–66	—	—
Ala-Ala-Ala	F	21–29	22	ND
Ala-Ala-Ala	R	23–27	22–28	ND
Met-Gly-Met	F	21–25	20	ND
Lys-Ala-Ala	F	7	—	—
Val-Val-Val	F	ND	—	—
Ala-Ala-Ala-Ala	F	ND	—	—
Ala-Ala-Ala-Ala-Ala	F	ND	—	—
Ala-Ala(P)	R	3	1–5	ND
Ala-Ala-Ala(P)	R	1	ND	ND
Ala-Ala-Ala-Ala(P)	R	1	ND	ND

ND, Not detectable; —, not determined.

Table 4. Competition for peptide uptake in strain ATCC 9790

Organisms were suspended in potassium phosphate buffer (pH 6.9, 50 mM) with glucose (0.2%, w/v) to a density of 0.10–0.21 mg dry wt ml⁻¹. Uptake of label from Ala-[¹⁴C]Ala and Ala-Ala-[¹⁴C]Ala was measured every 8 s for 40 s, and that from Ala-Ala-[¹⁴C]Ala(P) every 2 min for 8 min. Results are expressed as percentage inhibition of uptake in the presence of competitor relative to a control in its absence.

Substrate	Concn (mM)	Competitor	Concn (mM)	Percentage inhibition of uptake
Ala-[¹⁴ C]Ala	0.1	Ala-Ala-Ala	0.1	—8
Ala-[¹⁴ C]Ala	0.1	Ala-Ala-Ala	1.0	17
Ala-Ala-[¹⁴ C]Ala	0.1	Ala-Ala	0.1	74
Ala-Ala-[¹⁴ C]Ala(P)	0.1	Ala-Ala	0.1	26
Ala-Ala-[¹⁴ C]Ala(P)	0.1	Ala-Ala	1.0	89
Ala-Ala-[¹⁴ C]Ala(P)	0.1	Ala-Ala-Ala	0.1	72
Ala-Ala-[¹⁴ C]Ala(P)	0.1	Ala-Ala-Ala	1.0	96
Ala-Ala-[¹⁴ C]Ala(P)	0.1	Ala-Ala-Ala-Ala	0.1	78
Ala-Ala-[¹⁴ C]Ala(P)	0.1	Ala-Ala-Ala-Ala-Ala	0.1	69

high-rate transport system used for the uptake of natural dipeptides and tripeptides, of Ala-Ala(P) and of AX1. This system will be referred to as the dipeptide permease.

The results of competition studies for peptide uptake in strain ATCC 9790 are presented in Table 4. Ala-Ala was a much better inhibitor of the (rapid) uptake of Ala-Ala-Ala than Ala-Ala-Ala was of Ala-Ala uptake. This is consistent with the two peptides sharing a system (the dipeptide permease) with a much greater affinity for Ala-Ala. In contrast to its effect on Ala-Ala-Ala uptake, Ala-Ala was a poor inhibitor of the (slow) uptake of Ala-Ala-Ala(P), whereas Ala-Ala-Ala, Ala-Ala-Ala-Ala and Ala-Ala-Ala-Ala-Ala were good inhibitors. These results are, like those with the mutant strains reported above, consistent with the presence of a low-rate oligopeptide permease, and a high-rate dipeptide permease. Presumably the uptake of Ala-Ala-Ala, Ala-Ala-Ala-Ala and Ala-Ala-Ala-Ala-Ala via the oligopeptide system is not directly demonstrable because the low rates of uptake are undetectable by the fluorescence or

Table 5. *Uptake of peptides by different strains of S. faecalis*

Organisms were suspended in potassium phosphate buffer (pH 6.9, 50 mM) with glucose (0.2%, w/v) to a density of 0.11–0.27 mg dry wt ml⁻¹ and uptake was measured using the automated fluorescence (F) or radiotracer (R) assays. Results are the average of one to five determinations.

Peptide (0.1 mM)	Assay	Rate of uptake [nmol min ⁻¹ (mg dry wt) ⁻¹]		
		ATCC 9790	OG-FS5	NCIB 6459
Ala-Ala	R	99	44	52
Ala-Ala	F	95	52	—
Ala-Ala-Ala	R	25	94	14
Ala-Ala-Ala	F	25	82	—
Ala-Ala-Ala-Ala	R	ND	89	14
Ala-Ala-Ala-Ala	F	ND	110	—
Ala-Ala-Ala-Ala-Ala	F	ND	107	—
Ala-Ala(P)	R	3	12	—
Ala-Ala-Ala(P)	R	1	80	—
Ala-Ala-Ala-Ala(P)	R	1	80	—

ND, Not detectable; —, not determined.

radiotracer assays used. Uptake via this system is demonstrable only for those peptides in which the labelled moiety does not undergo exodus after cleavage (i.e. phosphonopeptides) (Table 3).

Strain differences in peptide uptake. The transport of homo-alanyl peptides was examined in three strains of *S. faecalis*, and differences in the relative rates of uptake of Ala-Ala, Ala-Ala-Ala and Ala-Ala-Ala-Ala were found (Table 5). Similarly, rates of uptake of phosphonopeptides by strain OG-FS5 showed differences from those found in strain ATCC 9790 (Table 5).

DISCUSSION

The demonstration of intact peptide accumulation by *S. faecalis* strain ATCC 9790 is good evidence for peptide transport being distinct from amino acid uptake. Extracellular cleavage, which would be necessary if residues from peptides were to be taken up as free amino acids, is not detectable, no cleavage or loss of peptide occurring with organisms deprived of a metabolizable energy source (Nisbet, 1980; Payne & Nisbet, 1981). Further evidence comes from the study of phosphonopeptide uptake; as the Ala(P) moiety is impermeant (Nisbet, 1980; Atherton *et al.*, 1979) the uptake of phosphonopeptides in *S. faecalis* must be in the peptide form. The presence of a peptide uptake system as such is therefore not in doubt.

The observed exodus of the constituent amino acids of peptides subsequent to uptake and cleavage has previously been demonstrated for *E. coli* (Payne & Bell, 1977, 1979), *Salmonella typhimurium* (J. W. Payne, unpublished observations) and *Staphylococcus aureus* (Perry & Abraham, 1979). This phenomenon presumably serves to regulate the intracellular pools of amino acids during peptide uptake (Payne, 1975).

The results of competition studies in the parent strain, and measurement of peptide transport in peptide analogue-resistant strains, point to the presence of two peptide permeases of differing specificity in *S. faecalis* ATCC 9790. The existence of a low-rate oligopeptide system is implied by the loss of uptake of Ala-Ala-Ala-Ala(P) and Ala-Ala-Ala(P) (both subject to inhibition by homo-alanyl oligopeptides) that accompanies acquisition of resistance to the toxic analogue Pro-Ala-aminooxypropionic acid (AX5) by the mutation to give strain PA 6102. The further presence of a high-rate system for dipeptides, and to a lesser extent tripeptides, is implied by the loss of uptake of all peptides transported by strain PA 6102, in the substrain PA 610201 selected as also being resistant to Ala-aminooxypropionic acid

(AX1). These results are not explicable by the presence of a single permease able to transport dipeptides and tripeptides, which handles oligopeptides after their extracellular cleavage. If this were the case, organisms resistant to AX1 (i.e. transport defective) should be isolated as readily from strain ATCC 9790 as from substrain PA 6102, whereas, in fact we have been completely unable to obtain such organisms from the former strain.

The C-terminal specificity of the two permeases in strain ATCC 9790 is different. The major dipeptide permease mediates the rapid uptake of Ala-Ala and Ala-Ala-Ala, but transports Ala-Ala(P) at only a low rate, and Ala-Ala-Ala(P) uptake is undetectable. In contrast the rate of uptake of Ala-Ala-Ala(P) and Ala-Ala-Ala-Ala(P) via the minor, oligopeptide permease seems comparable with that of their 'parent' peptides. This less strict C-terminal specificity of the oligopeptide permease compared with the dipeptide permease parallels the situation in *E. coli* (Payne & Gilvarg, 1978).

There are marked differences in the relative rates of peptide uptake by the three different strains of *S. faecalis* studied here. This could reflect either a different complement of permeases in these strains, or a similar array of permeases operating at different relative rates. Some evidence for the latter circumstance is found in different strains of *E. coli* (Payne & Bell, 1979). More detailed studies with transport-deficient mutants in other strains, together with competition experiments, are needed to clarify the point, but there is some evidence for two peptide permeases in strain OG-FS5 with similar specificity to those in strain ATCC 9790. Thus, Ala-Ala-Ala(P) and Ala-Ala-Ala-Ala(P) are transported at rates similar to their 'parent' peptides, whereas Ala-Ala(P) is taken up much more slowly than Ala-Ala, features which are observed respectively in the oligopeptide and dipeptide permeases of strain ATCC 9790.

The results presented here show that phosphonopeptides enter *S. faecalis* through the same permeases as natural peptides, as has previously been shown for *E. coli* (Ringrose & Lloyd, 1979). Evidence is also accumulating that a number of natural antimicrobial peptide analogues also use peptide permeases to gain access to their targets (Diddens *et al.*, 1979; Ringrose, 1980; Staskawicz & Panopoulos, 1980; Perry, 1981). The relatively broad specificity of the peptide permeases, particularly with respect to amino acid side chains and C-terminus (Payne & Gilvarg, 1978), make these systems particularly attractive for exploitation through the rational design of synthetic antibiotics, as exemplified by the phosphonopeptides.

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