Chemotaxis to Oligopeptides by *Pseudomonas aeruginosa*

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A number of peptides were evaluated as chemoattractants for *Pseudomonas aeruginosa*. Several strains recognized tri-, tetra-, penta-, and hexapeptides in a capillary tube assay. Tripeptides altered at the carboxyl terminus were good attractants, whereas tripeptides altered at the amino terminus did not serve as chemoattractants. Methionine-containing peptides were relatively poor attractants. Arginine-containing peptides gave the best responses. Reduced responses to larger peptides suggest that porin penetration is required. No extracellular peptidase activity was detected. We conclude that oligopeptides are good attractants and that specificity for chemotactic recognition of oligopeptides exists.

The capacity to search for nutrients and transport them effectively has enabled bacteria to grow and survive in a wide range of environmental conditions. Motility and chemotaxis are particularly important processes for gram-negative, free-living forms such as pseudomonads. Complex signaling processes have evolved which enable these bacteria to couple nutrient “sensing” with flagellar motor operation, enabling bacteria to swim up a chemical gradient (chemotaxis). In *Escherichia coli* and *Salmonella typhimurium*, for example, taxis systems that respond to 7 of 20 amino acids are highly developed (11), but specialized constitutive and individual systems have evolved for carbohydrates, such as glucose ribose, galactose, and maltose (14). In contrast, in *Pseudomonas aeruginosa*, many more amino acids are excellent attractants, particularly basic amino acids, such as arginine and glutamine (6, 17). Also, glucose taxis, for example, is strictly an inducible system, and glucose is a relatively poor attractant (17). Chemotaxis of *P. aeruginosa* to several amino acids is highly regulated in relation to the nitrogen source used for growth (6). Like enterics, pseudomonads appear to utilize methylated chemotaxis proteins as transducers (7).

Most gram-negative bacteria utilize small oligopeptides as both carbon and energy sources. Two important mechanisms involved in this process are transport and chemotaxis. Transport in *E. coli* and *S. typhimurium* has been characterized to a greater extent than that in *P. aeruginosa*. In these organisms, there exists a general peptide transport system with broad specificity, as well as a number of other transport systems that are restricted to certain substrates (2, 18). The most prominent uptake occurs via the oligopeptide permease, which provides the major pathway for peptides of up to five residues (9). However, several other specific peptide transport systems have been identified in enteric bacteria (2, 16). Chemotactic responses to dipeptides and weak chemotactic responses to tripeptides have been demonstrated. These responses require the *E. coli* Tap dipeptide transport system (15). In *P. aeruginosa*, it was shown that a variety of di-, tri-, tetra-, and pentapeptides served as sources of methionine for the growth of a methionine auxotroph. Since no peptidase activity was detected in the medium, it was concluded that an oligopeptide uptake system was operative (16). Further evidence indicated the presence of an oligopeptide permease in the inner membrane (10).

We report here that several strains of *P. aeruginosa* recognize a number of tripeptides, tetrapeptides, and several larger peptides as chemoattractants. The data indicate the presence of a well-developed response system for specific oligopeptides in pseudomonads.

*P. aeruginosa* PAO1, M2, and 170018, used in this study, were common laboratory strains. RM46 is a methionine auxotrophic mutant of strain PAO1 with a mutation located in the metIV transduction group (7, 16). RM9 is an arginine auxotrophic mutant of PAO1 (6, 16). For all experiments, bacteria were grown in mineral salts medium (6, 7). Mutants RM46 and RM9 were grown as previously described (6, 7). The amino acids were all of the L configuration and were purchased from Sigma Chemical Co., St. Louis, Mo. The peptides were also purchased from Sigma Chemical Co. or were a gift from Jeffrey Becker, University of Tennessee, Knoxville (16).

*P. aeruginosa* cells were grown to log phase and prepared for chemotaxis as previously described (6, 17). The swarming of motile cells into capillary tubes containing an attractant in CTX buffer was determined as previously described (7, 17), except that capillary tube assays were performed in chemotaxis chambers. Four chambers were made in a square Lucite plate measuring 5.5 cm per side and 1 cm thick. Each chamber consisted of two cylindrical compartments 7 mm in diameter and 5 mm in height, linked by a channel 24 mm long, 2 mm wide, and 2 mm deep (19). After cleaning, each chemotaxis chamber was placed in sterile petri plates and sterilized by exposure to UV radiation.

The chambers were placed on a slide warmer at 37°C, and each well was filled with 0.4 ml of the bacterial suspension described above. The amino acids and peptides used were each dissolved to a concentration of 10 μM in CTX buffer. Various amino acids or peptides were used to fill disposable capillary tubes (1 μl), and these were placed in channels of chambers containing the cell suspension (400 μl). Control capillaries contained CTX buffer alone and were used as indicators of background motility. Each capillary tube was carefully immersed, and the open-ended tips were centrally located in the chamber. After 40 min of incubation, the capillaries were withdrawn and washed with sterile water and the contents were expelled into tubes of saline. After appropriate dilutions, samples (0.1 ml) were plated in duplicate. Following incubation overnight at 37°C, the colonies were counted and the number of cells per capillary tube was

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calculated. Each amino acid, peptide, or control was run in duplicate chambers for each experiment, and each assay was performed in duplicate. The mean accumulation from all capillaries was expressed as the total number of cells per capillary. To normalize differences in motility, the chemotactic response was also calculated as a relative taxis response (RTR), which was the ratio of the accumulation in test capillaries to that in control capillaries. An RTR of 2 or greater was considered significant (1, 6, 17).

For peptidase assays, cells of PAO1 were grown to log and late log phases as previously described (6). The cells were centrifuged, and supernatant was obtained by filter sterilization. The supernatant (40 ml) was concentrated by lyophilization and then suspended in 5 ml of CTX buffer. The concentrated samples were dialyzed and again concentrated by lyophilization. The samples were suspended in 0.5 ml of CTX buffer. For each assay, 100-μl concentrated supernatant samples and 100 μl of a 10 mM solution of peptide or amino acids were mixed and incubated for 60 min at 37°C. After incubation, the samples were loaded onto cellulose paper and separated by thin-layer electrophoresis at pH 3.5 (23). The migration of the peptides and that of the free amino acid components were compared by fluorescamine staining and visualization under UV lamp.

To characterize the location of a peptidase, an osmotic-shock procedure was performed on PAO1 cells grown in mineral salts medium. This procedure was performed as described by Hoshino and Kageyama (12, 13). The supernatants from both the osmotic-shock buffer and the water extract were combined and centrifuged at 20,000 g for 10 min at 4°C. This supernatant was dialyzed against water and concentrated by lyophilization before analysis. To test for the presence of peptidase in different cell fractions, thin-layer electrophoresis was utilized to separate amino acids and peptides (5, 23).

Initially, the RTR of P. aeruginosa was determined for several amino acids by using the capillary tube assay. There was considerable RTR variation among the different amino acids. The RTR was expressed as the fold increase over the CTX buffer capillary control (RTR = 1.0), and a value above 2 was considered significant (6, 17). The amino acids arginine, leucine, and valine gave the strongest RTRs (>10.8, >10.6, and 8.4, respectively). Serine and methionine were considered good attractants, with RTRs of 3.8 and 3.4, respectively. Another good attractant is ω-aminoisobutyrate (7, 17). The RTR for glycine was 1.3. It is interesting that leucine and valine, along with other hydrophobic amino acids, are chemorepellants in E. coli (11) but strong attractants in P. aeruginosa.

The RTR of P. aeruginosa toward a group of peptides with various amino acid sequence lengths and compositions was tested. Some of the peptides were good chemoattractants, while others were poor chemoattractants. Initially, the response of P. aeruginosa PAO1 to a series of tripeptides, tetrapeptides, pentapeptides, and hexapeptides was determined. The RTRs are shown in Table 1. Peptides with different amino acid sequence sizes and compositions appeared to generate different RTRs. Generally, peptides containing arginine, such as the tripeptide Arg-Pro-Arg, the tetrapeptide Gly-Gly-Tyr-Arg, or the pentapeptide Ser-Asp-Gly-Arg-Gly, gave the strongest RTRs (RTRs, 3 to 6.0), while the tripeptide Ala-Gly-Ala gave a weaker RTR. The hexapeptide Lys-Arg-Thr-Leu-Arg-Gly gave a weaker RTR; however, the RTR value was significant. Compared with that obtained with arginine, the RTRs to the peptides Arg-Pro-Arg, Gly-Gly-Norleu, and Gly-Gly-Tyr-Arg were stronger.

<table>
<thead>
<tr>
<th>Attractant</th>
<th>CFU/capillary tube</th>
<th>RTR*</th>
<th>RTR with respect to Arg*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-Gly-Ala</td>
<td>1 × 10⁴</td>
<td>2.5</td>
<td>0.10</td>
</tr>
<tr>
<td>Arg-Pro-Arg</td>
<td>2.8 × 10⁵</td>
<td>3.0</td>
<td>1.20</td>
</tr>
<tr>
<td>Gly-Gly-Norleu</td>
<td>11.2 × 10⁵</td>
<td>6.1</td>
<td>1.34</td>
</tr>
<tr>
<td>Gly-Gly-Tyr-Arg</td>
<td>3.4 × 10⁵</td>
<td>6.0</td>
<td>1.70</td>
</tr>
<tr>
<td>Ser-Asp-Gly-Arg-Gly</td>
<td>1.9 × 10⁵</td>
<td>3.0</td>
<td>0.80</td>
</tr>
<tr>
<td>Lys-Arg-Thr-Leu</td>
<td>1.6 × 10⁵</td>
<td>2.5</td>
<td>0.76</td>
</tr>
<tr>
<td>Arg-Arg</td>
<td>1.4 × 10⁵</td>
<td>2.5</td>
<td>0.70</td>
</tr>
</tbody>
</table>

* Each was used at a concentration of 10 mM.
* RTR is expressed as the fold increase over the buffer capillary control. A value over 2 is considered significant.
* The RTR compared with that obtained with arginine is expressed as the fold increase or decrease in the peptide response.

An interesting finding was the significant RTR of 2.5 obtained for PAO1 to an 11-amino-acid peptide, angiotensin II (Ala-Pro-Gly-Asp-Ile-Tyr-Val-His-Pro-Phe). The molecular mass of angiotensin II is 1,243 g/mol. Although preliminary, these data suggested that specific peptides of greater than five to six amino acids are chemoattractants and that the ability of a peptide to penetrate the porin system is not necessarily a limitation for taxis as is seen with transport. However, specific porin recognition sites are sometimes utilized by distantly related compounds. In E. coli, a recently discovered cephalosporin entered the cell via the TonB-dependent iron system (3). Interestingly, evidence that a specific hormone-binding site for interleukin 1 exists in gram-negative bacteria is also accumulating (20). The capacity to bind this polypeptide may enhance virulence (20). The binding site for angiotensin remains to be determined.

In Fig. 1 are illustrated results from concentration response curves for two peptides and arginine. The peptides gave response profiles similar to those obtained for the amino acid.

**FIG. 1.** Comparative concentration response curves for chemotaxis of P. aeruginosa PAO1 to two peptides and arginine. Symbols: ○, Gly-Gly-Norleu; ▲, Arg-Pro-Arg; □, arginine.
To demonstrate that a strong response to arginine peptides is a general characteristic of *P. aeruginosa* strains, three additional strains were tested. Other *P. aeruginosa* strains, M2, 170018, and the *met* mutant RM46, used extensively in previous studies (6, 7) were tested for chemotactic responses to various peptides. As shown in Table 2, the tripeptide Arg-Pro-Arg was a strong chemoattractant (RTR, 3.5 and 10.6) for 170018 and M2, respectively, and the tripeptide Ala-Pro-Ala was a good attractant for 170018. When angiotensin II was tested with 170018, it gave a positive taxis response, comparable to that obtained with PAO1 (Table 1). The tetrapeptide Gly-Gly-Tyr-Arg was a good chemoattractant for *met* mutant RM46, as was the amino acid methionine. The arginine peptides tested were generally good attractants for all three of the strains evaluated.

Several peptides proved not to be chemoattractants. The heptapeptide Ala-Pro-Arg-Leu-Arg-Phe-Tyr did not produce a chemoattractant response with strain PAO1. The lack of response for the heptapeptide suggests that porin penetration is required. Also, a 13-amino-acid peptide (Asp-Ser-Arg-Ser-Arg-Val-Leu-Arg-Tyr-Leu-Leu-Glu-Ala-Lys-Glu) with a molecular mass of 1,611 g/mol was not an attractant. These results indicate that in general, like transport, the ability of a peptide to penetrate the porin system is necessary for taxis. These data are in contrast to the angiotensin data, which suggest that a surface binding site for angiotensin exists.

Capillary tube assays were performed with blocked amino acids to determine some of the structural requirements necessary for recognition. Peptides with a C-terminal amino acid analog (norleucine) were recognized (Table 1); however, an N-terminally blocked peptide (Bloc-Gly-Gly-Met) and peptides with an N-terminal amino acid analog (Norleu-Gly-Gly) were not chemoattractants. These initial data suggest that recognition for taxis exhibits the same requirements as transport, as shown with strain RM46 by Miller and Becker (16). However, additional experiments showed that two tripeptides (Met-Gly-Gly and Gly-Met-Gly) and a six-amino-acid peptide (Gly-Tyr-Gly-Gly-Phe-Met) were poor chemoattractants to PAO1 (RTRs, 0.6 to 1.0). When the responses to the Met-containing peptides (Ac-Met-Met-Met, Met-Gly-Gly, Gly-Met-Gly, and Met-Leu-Met) were tested with RM46, the results showed that they were also poor attractants. However, Met was a good chemoattractant for RM46 (Table 2).

It appears that methionine-containing peptides, although an excellent source of Met for *P. aeruginosa* transport and growth (16), are poor attractants, even though methionine itself is an attractant. We confirmed that RM46, a Met-requiring mutant, grew well with Met-Gly-Gly as a Met source (16), even though this peptide was not an attractant. On the basis of these results, it does not appear that chemotaxis response levels are necessarily coordinated with oligopeptide transport. Experimental evidence indicates that Met-containing peptides may be utilized for growth in place of Met by RM46 (16); however, Met-containing peptides are considered poor chemoattractants by our assay methods. On the other hand, Arg mutant RM9 was shown to require Arg or an Arg-containing peptide for growth (data not shown), and the latter is a good attractant. Thus, it is not clear whether some chemotaxis and transport pathways have components in common.

In *P. aeruginosa*, it has been shown by using a methionine mutant (RM46) that a variety of di-, tri-, tetra-, and pentapeptides serve as sources of methionine for growth. Since no peptidase activity was detected either in the medium or in the periplasm (10, 16), it was concluded that an oligopeptide uptake system was operative.

Although peptidase degradation is unlikely to be a factor involved in conditions for the capillary tube assay (bacteria are incubated for 30 to 40 min in CTX buffer), it was important to establish whether there was some peptide breakdown that could contribute at least in part, to the attractant response. Miller and Becker (16) have previously reported that no oligopeptidase was present in their growth medium for strain RM46. We examined the release of amino acids from tripeptides by staining with fluorescamine and examination under UV light (Fig. 2). Specifically, concentrated growth medium from PAO1 cells grown to either log or late phase was prepared as described in Materials and Methods and suspended in a small volume of CTX buffer. Equal volumes of medium samples were mixed with substrate and incubated for 60 min at 37°C. The reaction samples were separated by cellulose thin-layer electrophoresis. Peptides and free amino acid components were detected. 

### Table 2. Responses of different strains of *P. aeruginosa* to peptide chemoattractants

<table>
<thead>
<tr>
<th>Strain, attractant</th>
<th>CFU/capillary tube</th>
<th>RTR</th>
<th>RTR with respect to Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>M2, Ala-Gly-Ala</td>
<td>7.0 x 10^2</td>
<td>3.5</td>
<td>1.1</td>
</tr>
<tr>
<td>M2, Arg-Pro-Arg</td>
<td>7.0 x 10^2</td>
<td>3.5</td>
<td>1.1</td>
</tr>
<tr>
<td>170018, Ala-Gly-Ala</td>
<td>1.5 x 10^2</td>
<td>2.3</td>
<td>2.8</td>
</tr>
<tr>
<td>170018, Arg-Pro-Arg</td>
<td>6.8 x 10^2</td>
<td>10.6</td>
<td>4.5</td>
</tr>
<tr>
<td>170018, angiotensin II</td>
<td>5.0 x 10^2</td>
<td>2.5</td>
<td>0.34</td>
</tr>
<tr>
<td>RM46, Met</td>
<td>7.1 x 10^3</td>
<td>3.4</td>
<td>1.1</td>
</tr>
<tr>
<td>RM46, Gly-Gly-Tyr-Arg</td>
<td>2.5 x 10^3</td>
<td>11.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Data were obtained and computed as detailed in the footnotes to Table 1.
  \( ^* \) Each attractant was used at a concentration of 10 mM.

\( ^\text{a} \) L-amino-acid peptide (molecular mass, 1,243 g/mol) (Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe).

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**FIG. 2.** Assays to detect peptidase activity in extracellular medium from *P. aeruginosa* grown to mid or late log phase. Peptides and amino acids were separated by thin-layer electrophoresis on cellulose as described in Materials and Methods. Following detection with fluorescamine, spots were outlined. Lanes: A, peptide (Gly-Gly-Norleu); B, extracellular medium without the peptide; C, extracellular medium from log-phase cells with the peptide; D, glycine and norleucine; E, extracellular medium with the peptide from late-log-phase cells; F, glycine.
by fluorescein staining. As shown in Fig. 2, the absence of release of free amino acids (lanes C and E) indicated that no peptidase activity was associated with extracellular medium from cells grown to either the log or the late log phase.

To determine whether peptidase activity was associated with the periplasm, log-phase PA01 cells were osmotically shocked and the concentrated periplasmic fraction derived from this procedure was utilized in a peptidase experiment as described above. Following thin-layer electrophoresis analysis and fluorescein staining, similar results were seen (data not shown). No peptidase activity was detected in the released periplasm of osmotically shocked PA01 cells grown to the log phase. The lack of detectable protease activity in the extracellular spent medium and in periplasmic fluids from osmotically shocked cells is consistent with the requirement for an intact peptide for recognition. It can be inferred from these data that the processing of oligopeptide peptidase is located on the inner membrane, as previously reported (10).

There have been few studies of taxis to oligopeptides, probably because most-studied group, the enterics, show only weak responses to tripeptides. The experiments on taxis to oligopeptides presented here were compared with responses obtained with arginine as a positive control, since it is one of the best attractants for _P. aeruginosa_ (6). With several different strains, good taxis responses were obtained with a number of peptides and tetrapeptides. Arginine-containing peptides were particularly good attractants for all of the strains tested. In some cases, arginine-containing peptides surpassed arginine in producing an RTR. Peptides such as Ala-Gly-Ala also gave a good response, but peptides containing methionine were poor attractants. The latter result was unexpected; since methionine is a reasonably good attractant, its methyl group is needed for chemotaxis (7, 17), and methionine peptides are effectively transported (16). The poor response of _P. aeruginosa_ to Met-containing peptides suggests that their chemotaxis and transport are not necessarily coupled, or at least that Met-containing peptides are recognized by several divergent pathways in pseudomonads. It is interesting that _P. aeruginosa_ excretes methionine-containing peptides termed chemotactins which serve as attractants for phagocytes (8). Thus, for chemotaxis, methionine-containing peptides may represent a separate physiological class of excreted oligopeptides which could not, therefore, serve as attractants. These peptides might relate to some other required signaling function.

With respect to size limitations, alanine-glycine and arginine-containing tri- and tetrapeptides were good attractants. An arginine-containing pentapeptide and hexapeptide were recognized, but not as well as the smaller arginine-containing oligopeptides. An arginine-containing heptapeptide (Ala-Pro-Arg-Leu-Arg-Phe-Tyr) was not an attractant in our system. On a relative basis, therefore, as peptide length approaches six to seven amino acids, taxis is severely reduced. This suggests that functional porins are required for chemotaxis. The recognition specificity observed, including a size requirement; lack of response to methionine-containing peptides; and the requirement for a recognizable N terminus but not a C terminus together indicate that specified chemical motifs are present in oligopeptide attractants.

The increased response to arginine-containing peptides observed emphasizes the apparent importance for _P. aeruginosa_ of acquiring basic amino acids and peptides. Of relevance to the results reported here is the recent finding that resistance to imipenem (21) correlates with the absence of a specific _D_ porin in _P. aeruginosa_ (21, 22). Further investigation has shown that imipenem structurally resembles basic amino acids and small peptides and that the _D_ porin greatly enhances the specific recognition and entrance of basic amino acids and peptides through the outer membrane (22). Thus, efficient operation of active transport of these compounds requires the _D_ porin. It is very likely that the _D_ porin enhances taxis to basic amino acids and peptides (4). We are currently testing this hypothesis.

REFERENCES