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Specificity of Cellular DNA-Binding Sites of Microbial Populations in a Florida Reservoir

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The substrate specificity of the DNA-binding mechanism(s) of bacteria in a Florida reservoir was investigated in short- and long-term uptake studies with radiolabeled DNA and unlabeled competitors. Thymine oligonucleotides ranging in size from 2 base pairs to 19 to 24 base pairs inhibited DNA binding in 20-min incubations by 43 to 77%. Deoxynucleoside monophosphates, thymidine, and thymine had little effect on short-term DNA binding, although several of these compounds inhibited the uptake of the radiolabel from DNA in 4-h incubations. Inorganic phosphate and glucose-1-phosphate inhibited neither short- nor long-term binding of [^{3}H]- or [^{32}P]DNA, indicating that DNA was not utilized as a phosphorous source in this reservoir. RNA inhibited both short- and long-term radiolabeled DNA uptake as effectively as unlabeled DNA. Collectively these results indicate that aquatic bacteria possess a generalized nucleic acid uptake/binding mechanism specific for compounds containing phosphodiester bonds and capable of recognizing oligonucleotides as short as dinucleotides. This binding site is distinct from nucleoside-, nucleotide-, phosphomonoester-, and inorganic phosphate-binding sites. Such a nucleic acid-binding mechanism may have evolved for the utilization of extracellular DNA (and perhaps RNA), which is abundant in many marine and freshwater environments.

High-molecular-weight dissolved organic compounds are abundant in freshwater and marine ecosystems (2, 14, 15). These materials can be in the form of combined amino acids (1), polysaccharides (4), or nucleic acids (5, 15) or in the form of more refractory materials such as lignins (7), humic acids, and phenols (3, 6).

Although biologically labile, dissolved macromolecules such as DNA and protein are known to be utilized by populations of aquatic bacteria, mechanisms of uptake for the most part are not understood. Hollibaugh and Azam (8) found that amino acids derived from proteins were taken up by marine bacteria by mechanisms distinct from those for free amino acids. The lack of extracellular proteolytic activity indicated that hydrolysis occurred at the cell surface.

DNA-binding and -uptake mechanisms have been most widely studied in naturally transformable bacteria (19). In gram-positive microorganisms such as *Bacillus subtilis* and *Streptococcus pneumoniae*, double-stranded DNA is bound, regardless of source, whereas RNA, DNA:RNA hybrids, and single-stranded DNA are not bound (20). For strains of *S. pneumoniae* and *Streptococcus sanguis*, DNA-binding proteins have been described and may have endonuclease activity (20). Divalent cations have been shown to be required for DNA binding in these organisms.

DNA binding in gram-negative bacteria such as *Haemophilus* spp. is specific for particular DNA sequences. There is no requirement for divalent cations, and DNA enters the cell in a double-stranded form (16). Specialized membrane residues termed transformasomes (9), which facilitate DNA transport into the cell, have been described in *Haemophilus* spp.

The presence of cell-associated and extracellular DNases in marine waters (10–12, 18) might suggest that the primary mechanism of DNA utilization is hydrolysis, followed by uptake of nucleosides. Previous research on the utilization of DNA by estuarine bacteria indicated that rapid binding of DNA was at sites other than those specific for nucleoside and nucleotide binding (17). This was followed by hydrolysis of bound DNA and uptake of nucleic acid moieties by nucleoside-/nucleotide-specific binding sites (17).

The purpose of the present study was to determine the specificity of DNA-binding sites in natural populations of freshwater bacteria. Our results indicate the presence of nucleic acid-specific receptors in these populations which are different from nucleotide-/nucleoside-binding sites and inorganic phosphate-transport sites.

MATERIALS AND METHODS

Labeling of DNA. [^{3}H]DNA was prepared by end labeling *Hin*dIII-digested λ DNA with all four [^{3}H]deoxynucleoside triphosphates as previously described (17). ^{32}P-end-labeled DNA was prepared by the method of Maniatis et al. (13), with the following modifications. Reaction mixtures contained *Hin*dIII λ template DNA (0.5 μg), 4 μl each of [α-^{32}P]-labeled dTTP, dGTP, dCTP, and dATP (each 3,000 Ci/mmol, 10 μCi/ml; DuPont, NEN Research Products, Boston, Mass.), 5.7 μM (final concentration) unlabeled dATP, dGTP, dCTP, and dTTP, 3.5 μl of 10× nick translation buffer (13), and 3.6 U of large-fragment DNA polymerase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in a 35-μl total volume. This procedure resulted in [^{32}P]DNA with a specific activity similar to that of [^{3}H]DNA (i.e., 30 to 60 μCi/μg of DNA).

Sampling site. All samples were collected from the surface waters of the Medard Reservoir, Valrico, Fla.

Uptake of [^{32}P]DNA and [^{3}H]DNA by natural microbial populations. To determine similarities and differences in the capability of natural populations to take up phosphate-labeled and base-labeled DNA, 35 ml of Medard Reservoir water was amended with [^{32}P]DNA (0.1 μCi/ml) or [^{3}H]DNA (0.26 μCi/ml). Triplicate 2-ml samples were filtered onto 0.2-μm Nuclepore filters (Nuclepore Corp., Pleasanton, Calif.) mounted on sidearm collection tubes, and the filters were washed with 3 ml of 10 mM Tris hydrochloride–100

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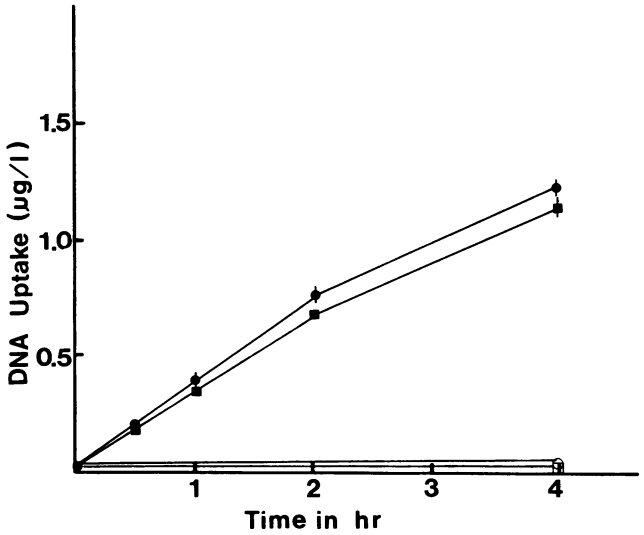

FIG. 1. Comparison of uptake of [^{3}H]DNA (circles) and [^{32}P] DNA (squares) by populations of bacteria in the Medard Reservoir. Open symbols designate radiolabeled-DNA binding in autoclaved, sterile, filtered controls.

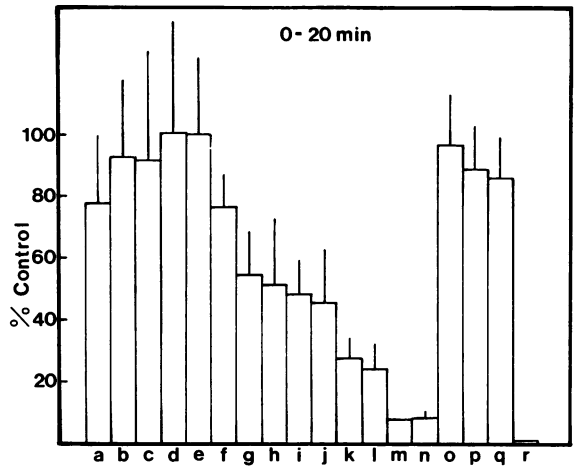

FIG. 2. Short-term uptake of labeled DNA by natural populations of freshwater bacteria in the presence of unlabeled competitors. Results are expressed as percentages of the rate for the no-inhibitor control. a, dTMP; b, dAMP; c, dCMP; d, dGMP; e, thymidine; f, thymine; g, oligothymidylic acid dT_2; h, dT_3; i, dT_4; j, dT_5; k, dT_{10}; l, dT_{19-24}; m, RNA; n, DNA; o, 5 µM sodium phosphate; p, 30 µM sodium phosphate; q, glucose-1-phosphate; r, autoclaved, sterile, filtered control.

mM NaCl–1 mM EDTA, pH 7.2, containing 10 µg of DNA per ml (DNA wash) and counted for radioactivity by liquid scintillation counting. The filtrates were added to glass test tubes containing 0.5 ml of 100% trichloroacetic acid (TCA), chilled for at least 30 min, and filtered onto 0.2-µm Nuclepore filters. The filters were washed with 5.0 ml of 5% TCA and then counted for radioactivity by liquid scintillation counting. A 1-ml portion of this filtrate was also counted for radioactivity by liquid scintillation counting. Samples were taken at 0, 0.5, 1, 2, and 4 h.

Competition for DNA binding by unlabeled compounds. A 20- or 30-ml sample of water was added to an acid-cleaned, sterile polymethylpentene flask, and the unlabeled competitor (5 µM deoxynucleoside monophosphate [dNMP], thymidine, or thymine [equivalent to 1 to 2 µg/ml], glucose-1-phosphate at 5 µM, oligonucleotides at 1 µg/ml, RNA and DNA at 1 or 1.5 µg/ml, orthophosphate at 5 or 30 µM) was then added. Immediately thereafter [^{3}H]DNA or [^{32}P]DNA was added. Triplicate (2- or 3-ml) samples were taken within 15 s, at 20 min, and at 4 h and were filtered onto 0.2-µm Nuclepore filters, and the filters were washed with 3.0 ml of DNA wash to stop the radiochemical reaction. Positive controls consisted of sample water receiving no competitor but an equal volume of the buffer used to dissolve the competitor (usually 20 or 30 µl of sterile TE [13]), while negative controls were autoclaved, sterile, filtered sample water.

RESULTS AND DISCUSSION

Figure 1 displays the results of a time course study on the uptake of [^{3}H]DNA or [^{32}P]DNA by ambient microbial populations in the Medard Reservoir. The uptake rates for [^{3}H]DNA and [^{32}P]DNA were similar, indicating that either isotopically labeled DNA would be appropriate for competition studies. Rates of DNA hydrolysis, as indicated by the production of TCA-soluble material over 4 h, were also similar (0.41 µg of DNA fragments per liter per h for [^{3}H]DNA and 0.44 µg/liter per h for [^{32}P]DNA, data not shown). These forms of DNA were labeled in different components, the tritium being solely in the base moieties and the phosphorous in the diester backbone. If DNA was first hydrolyzed and hydrolysis was followed by nucleoside uptake, we might expect a greater proportion of [^{32}P]DNA in the TCA-soluble material and less in the cell-associated fraction than for [^{3}H]DNA. Since uptake rates and production of soluble extracellular radioactivity were similar for both isotopes, it seems unlikely that DNA was hydrolyzed and dephosphorylated before binding. These results argue for the binding of DNA by cells rather than binding of the hydrolyzed components only.

The results of short-term binding of [^{3}H]DNA by natural populations in the presence of various unlabeled competitors appears in Fig. 2. This figure contains the cumulative data from more than 108 individual incubations performed from 16 samplings from 1986 to 1989. All data are presented as percentages of the rate for the no-inhibitor control, because actual rates (in micrograms of DNA per liter per hour) varied between samplings. Nucleoside monophosphates, thymidine, thymine, glucose-1-phosphate, and inorganic phosphate yielded similar rates of DNA uptake, rates that averaged between 80 and 100% of that for the no-inhibitor control.

The oligonucleotides dT_2, dT_3, dT_4, and dT_5 inhibited DNA binding by 43.2 to 55.4% (Fig. 2). The longer oligonucleotides dT_{10} and dT_{19-24} inhibited DNA binding by 74.2 and 76.9%, respectively. RNA inhibited [^{3}H]DNA binding as effectively as calf thymus DNA did (both 93.5% inhibition).

The data were transformed by using the arcsine transformation (21) for statistical testing, because percentage data are usually not normally distributed. Analysis of variance (ANOVA; 21) and multiple-range testing of the arcsine-transformed data indicated that the deoxynucleotides, thymidine, thymine, inorganic phosphate, and glucose-1-phosphate all yielded statistically similar rates of DNA binding (Table 1) that often overlapped the no-inhibitor control (Fig. 2). DNA-uptake rates in the presence of the oligonucleotides were significantly lower than those in the presence of nucleotides, nucleosides, and other phosphorylated compounds

TABLE 1. Statistical comparison of arcsine-transformed data[a]

Uptake	Substrate and rank																	
Short-term																		
Rank	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Treatment[b]	AFSCon	DNA	RNA	dT 19-24	dT 10	dT 5	dT 4	dT 3	dT 2	Thy	dTmP	G1P	P30	dAMP	dCMP	P5	TdR	GMP
Long-term																		
Rank	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Treatment	AFSCon	RNA	DNA	dT 19-24	dT 10	dT 5	dT 4	dT 3	dT 2	dAMP	dCMP	dTMP	Thy	P30	TdR	dGMP	G1P	P5

[a] Results of multiple-range test of 103 individual treatments after analysis of variance testing showed that the means were not all the same. Underlined treatments are statistically similar at the 95% confidence interval.

[b] Abbreviations are as follows: AFSCon, autoclaved, filtered, sterilized control; RNA, wheat germ RNA; DNA, calf thymus DNA; dT_{19-24}, dT_{10}, dT_5, and dT_2, polymers of oligothymidylic acid; dTMP, deoxythymidine monophosphate; Thy, thymidine; G1P, glucose-1-phosphate; dAMP, deoxyadenine monophosphate; P30, 30 μM sodium phosphate; P5, 5 μM sodium phosphate; dGMP, deoxyguanosine monophosphate; TdR, thymidine; and dCMP, deoxycytidine monophosphate.

(Table 1), indicating that oligonucleotides significantly inhibited short-term DNA binding. RNA and calf thymus DNA inhibited [^{3}H]DNA binding to a greater degree than oligonucleotides dT_2 through dT_5 and were as effective as dT_{10} and dT_{19-24}.

To determine if DNA binding was simply a phosphate-charge interaction similar to that of DNA with hydroxylapatite, several of the experiments were repeated by using [^{32}P]DNA. Uptake rates in the presence of 5 and 30 μM inorganic phosphate and glucose-1-phosphate were similar to those of controls (92 ± 7%, 88 ± 20%, and 97 ± 2.3% of the no-inhibitor control rate, respectively [data not shown]). Nucleotides and thymidine also had no significant effect on [^{32}P]DNA binding, while oligonucleotides were not investigated. These results indicated that inorganic phosphate-binding mechanisms or monophosphate ester-binding sites are unique and different from DNA-binding sites.

The effect of the above competitors on long-term binding and uptake of radioactivity from [^{3}H]DNA was also investigated. Interpretation of long-term uptake studies are difficult because both the labeled DNA and polymeric inhibitors (i.e., oligonucleotides, RNA, and DNA) become hydrolyzed during the incubation period (17). Thus, accumulation of radioactivity might result from uptake of DNA hydrolysis products (nucleosides, etc.) rather than DNA itself. Several transport processes might be involved, and effects of competitors may be less easily interpreted. In light of these caveats, several observations are still worth noting. Compounds which strongly inhibited short-term binding of DNA (i.e., oligonucleotides, RNA, and calf thymus DNA) also inhibited long-term uptake of radioactivity from [^{3}H]DNA (Fig. 3). As with short-term uptake, inorganic phosphate and glucose-1-phosphate had no effect on long-term DNA uptake, suggesting that DNA was not utilized as a phosphorous source. The effect of nucleotides on long-term DNA uptake was variable, with dTMP, dAMP, and dCMP significantly inhibiting DNA binding, while dGMP and thymidine apparently did not inhibit it.

Previous research on DNA-uptake mechanisms in estuarine bacteria has indicated that nucleosides, nucleotides, and bases had little effect on DNA binding in 20-min incubations (17), as observed in the present study. In long-term incubations, these compounds inhibited uptake of radioactivity from [^{3}H]DNA by nearly 75%. This was attributed to hydrolysis of DNA, followed by uptake of nucleosides (17). A greater amount of extracellular TCA-soluble radioactive material was produced in the presence of dNMPs (17), further supporting the uptake of nucleotides from hydrolyzed DNA.

A possible explanation for the differences in 4-h DNA-uptake inhibition patterns between marine and freshwater bacterial populations observed in this study may be variability in levels of DNase in the latter environment. Thus, during certain samplings, if DNA was not rapidly hydrolyzed,

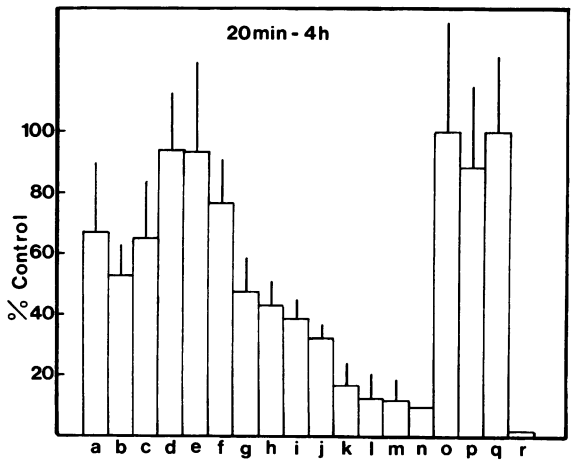

FIG. 3. Long-term binding (20 min to 4 h) of labeled DNA by natural populations of freshwater bacteria. Letter designations of competitors are as in the legend to Fig. 2.

long-term (4-h) inhibition of DNA uptake by nucleosides might not be expected to occur. Stable extracellular DNase activity has been found in marine waters (10, 12, 18). High levels of magnesium ions, as found in seawater, also favor DNase activity, since Mg^{2+} is a cofactor for DNase.

It may be argued that results obtained with the dNMPs are not strictly comparable to those obtained with the oligonucleotides, since the former were added at a concentration of 5 μM and the latter were added at concentrations of 1 to 1.5 μg/ml. A 5 μM concentration of the dNMPs is approximately 1.4 to 1.8 μg/ml by weight. For oligonucleotides, keeping a constant molarity would have been difficult for the large oligonucleotides and impossible for RNA or calf thymus DNA. Thus, for convenience, these were added at 1 or 1.5 μg/ml. For dT_2, this corresponds to 1.5 μM, but for dT_{19-24}, it corresponds to 0.13 μM. Since the latter strongly inhibited DNA binding in spite of the lower concentration, we feel that our approach was justified. These competitors were added in excess of radiolabeled DNA by factors of 66- to 150-fold (wt/wt).

Our results indicate a requirement for a phosphodiester bond for recognition by the DNA-binding site. Apparently one phosphodiester bond (as in the dinucleotide form of oligothymidylic acid) was sufficient to inhibit short-term DNA binding significantly. Phosphomonoester bonds had little effect on rapid DNA binding. The short-term binding site apparently possesses a broad enough specificity for RNA, DNA, and single-stranded DNA (as in oligonucleotides). The site is also apparently different from the inorganic phosphate-transport site. Additionally, our results indicate that DNA was not used as a phosphate source by bacteria in this reservoir. This does not preclude the use of DNA as a phosphorous source in other environments (particularly low-phosphate or phosphate-limited environments).

These studies imply the evolution of specific nucleic acid-binding mechanisms in aquatic bacteria. These have most likely evolved for the utilization of extracellular DNA, which we have found in abundant quantities in aquatic environments (5).

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