Previous data indicated that *Pseudomonas aeruginosa* exoenzyme S (ExoS) ADP-ribosylated Ras at multiple sites. One site appeared to be Arg41, but the second site could not be localized. In this study, the sites of ADP-ribosylation of c-Ha-Ras by ExoS were directly determined. Under saturating conditions, ExoS ADP-ribosylated Ras to a stoichiometry of 2 mol of ADP-ribose incorporated per mol of Ras. Nucleotide occupancy did not influence the stoichiometry or velocity of ADP-ribosylation of Ras by ExoS. Edman degradation and mass spectrometry of V8 protease generated peptides of ADP-ribosylated Ras identified the sites of ADP-ribosylation to be Arg41 and Arg128. ExoS ADP-ribosylated the double mutant, RasR41K,R128K, to a stoichiometry of 1 mol of ADP-ribose incorporated per mol of Ras, which indicated that Ras possessed an alternative site of ADP-ribosylation. The alternative site of ADP-ribosylation on Ras was identified as Arg38, which was on the same α-helix as Arg128.

Arg41 and Arg128 are located within two different secondary structure motifs, β-sheet and α-helix, respectively, and are spatially separated within the three-dimensional structure of Ras. The fact that ExoS could ADP-ribosylate a target protein at multiple sites, along with earlier observations that ExoS could ADP-ribosylate numerous target proteins, were properties that have been attributed to several vertebrate ADP-ribosyltransferases. This prompted a detailed alignment study which showed that the catalytic domain of ExoS possessed considerably more primary amino acid homology with the vertebrate mono-ADP-ribosyltransferases than the bacterial ADP-ribosyltransferases. These data are consistent with the hypothesis that ExoS may represent an evolutionary link between bacterial and vertebrate mono-ADP-ribosyltransferases.

Cystic fibrosis patients, burn wound victims, and the immunocompromised are particularly susceptible to infection by *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen (1). A number of virulence determinants contribute to the pathogenesis of *P. aeruginosa*, including two ADP-ribosyltransferases, Exotoxin A and Exoenzyme S (ExoS) (2). ExoS is a 49-kDa protein (3) that is secreted by the type III mechanism of *P. aeruginosa* (4), and ADP-ribosylates a number of target proteins in vitro including apolipoprotein A1, IgG3 (5), vimentin (6), and several members of the Ras superfamily (7). In vivo, ExoS is a cytotoxin (8) that ADP-ribosylates Ras during the course of infection in cultured cells (9), disrupting Ras-mediated signal transduction (10). Indirect methods were used to determine that Exoenzyme S ADP-ribosylates c-Ha-Ras at multiple sites (10).

Several vertebrate ADP-ribosyltransferases have been identified to date. The vertebrate ADP-ribosyltransferases possess specific properties that are distinct from the bacterial ADP-ribosyltransferases. Several of the vertebrate ADP-ribosyltransferases have the capacity to ADP-ribosylate multiple target proteins. For example, a murine lymphocyte transferase ADP-ribosylates a number of cell surface proteins (11), whereas rabbit skeletal muscle ADP-ribosyltransferase ADP-ribosylates several proteins in skeletal muscle T-tubules (12). In addition, several vertebrate ADP-ribosyltransferases modify target proteins at multiple sites. Both turkey erythrocyte type A ADP-ribosyltransferase (13) and chicken ADP-ribosyltransferase (14) ADP-ribosylate actin at two arginine residues.

These sites of ADP-ribosylation are located in different regions of actin, which suggests that each ADP-ribosylation event is independent. Although the role of endogenous ADP-ribosylation in the regulation of eukaryotic cell physiology has not been defined in detail, the RT6 transferase appears to be involved in the regulation of T-lymphocyte function (15), and endogenous ADP-ribosylation may contribute to hippocampal long-term potentiation (16).

In this study, the ADP-ribosylation of Ras by Exoenzyme S was characterized. Using both mass spectrometry and rpHPLC coupled with Edman degradation, it was determined that ExoS ADP-ribosylates Ras at arginines 41 and 128. These residues are located in a β-sheet and α-helix, respectively, which are spatially separated in Ras. Thus, ExoS has functional similarities to the eukaryotic ADP-ribosyltransferases because ExoS ADP-ribosylates a number of target proteins in vitro and ADP-ribosylates Ras at two nonadjacent arginine residues. In addition, homology studies have identified more primary sequence homology between ExoS and the vertebrate ADP-ribosyltransferases than the bacterial ADP-ribosyltransferases.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents were purchased: [adenylate phosphorothioate]NAD from NEN Life Science Products; Sculptor in vitro mu...
tagesis kit from Amersham Pharmacia Biotech, bovine serum albumin from Pierce Biochemicals, DNA oligonucleotides from Operon, and GTP·S from Sigma. Recombinant factor activating exoenzymes (FAS) and a c-Ha-Ras vector were gifts from H. Fu (Emory University).

**Purification of His-tagged Ras Proteins—** Recombinant His-tagged Ras was expressed in *Escherichia coli* and purified by Ni²⁺ affinity chromatography as described previously (10) with several modifications. His-tagged Ras proteins were eluted from the Ni²⁺ affinity resin in elution buffer containing 3 μM GTP and 10 mM MgCl₂. Eluted proteins were dialyzed into buffer (20 mM Tris-HCl (pH 7.6), 10 mM EDTA, 5 mM MgCl₂, 1 mM dithiothreitol, and 10% glycerol) and stored at −70 °C. These conditions were optimal for exchange of bound nucleotide from Ras proteins.

**Nucleotide Loading of Ras Proteins—** 20 μM Ras was incubated alone or with 1 mM of either GTP·S or GDP for 30 min at 30 °C. Reactions were stopped by the addition of 20 mM MgCl₂. The stoichiometry of nucleotide loading of Ras was monitored radioanalytically, using [³⁵S]GTP·S.

**ADP-ribosylation of the Ras Proteins by ExoS—** Reaction mixtures contained (25 μl): 0.2 M sodium acetate (pH 6.0), 50 μM [adenylate phosphate-³⁵P]NAD (specific activity 0.25 μCi per 1.25 nmol NAD), 5 μM Ras or Ras-SAAX, FAS, and ΔN222 (a catalytic, deletion peptide of ExoS). FAS and ΔN222 were added in equivalent amounts. Reactions were stopped at the indicated times by spotting an aliquot of the reaction mixture onto acetic acid-saturated Whatman 3-mm paper. The papers were washed three times for 10 min each in 7.5% trichloroacetic acid, and radioactivity was quantitated by scintillation counting. Stoichiometry of ADP-ribosylation was determined as the moles of ADP-ribosylated/mole of Ras. Velocity of ADP-ribosylation was determined by linear regression analysis. Ras-SAAX is a deletion peptide of Ras where the four C-terminal amino acids have been deleted, which eliminates its capacity to be acylated, making it more amenable to biochemical manipulation.

**Identification of the Sites of ADP-ribosylation within Ras—** Ras-SAAX or Ras-SAAX-R41K,R128K (20 μM) was incubated with 0.4 μM ΔN222, 400 μM NAD, and 1.2 μM FAS for 2 h at room temperature. Stoichiometry of ADP-ribosylated Ras was determined by subjecting an aliquot of the reaction mixture to SDS-polyacrylamide gel electrophoresis and subjecting the protein band corresponding to Ras to scintillation counting. Following ADP-ribosylation, the reaction mixture was dialyzed overnight into 10 mM Tris-HCl (pH 7.6), containing 20 mM NaCl, to remove unreacted NAD. Dialyzed samples were distributed into aliquots and lyophilized. Lyophilized samples were resuspended in 50 mM NH₄Ac (pH 4.0), and *Staphylococcus aureus* V8 protease was added at a w:w ratio of 1:10 (protease:Ras). Samples were digested overnight, lyophilized, and suspended in 0.1% trifluoroacetic acid. The digested degradation procedure. Hydrophilic phenyl isothiocyanate-modified amino acids will not be extracted with butyl acetate from the glass fiber filter during Edman degradation as shown in the case of cysteine.² The fact that radioactivity was recovered on the glass fiber filter but not in the eluate of the Edman degradation is consistent with the idea that ADP-ribosylated arginine was not extracted from the glass fiber filter during cycle 4, and Arg⁴¹ is one site of ADP-ribosylation. The first ten amino acids of the 48-min fraction contained a peptide corresponding to amino acids 127–136 of Ras, which included Arg¹²⁸ and Arg¹³⁵ (Table I). Amino acid yields at each cycle were similar (within about 2-fold), with the exception of cycle 2 where there was a reduction in the yield of the predicted amino acid, Arg¹²⁸. This was consistent with Arg¹²⁸ having been ADP-ribosylated. The high yield of Arg at cycle 9 indicated that Arg¹³⁵ had not been ADP-ribosylated and indicated that the reduction in yield seen in cycle 2 of the 48-min fraction and cycle 4 of the 24-min fraction was not because of a generalized decrease in yield of arginine after Edman degradation.

The sites that ExoS ADP-ribosylated on wild-type Ras were also determined by mass spectrometry. Ras alone or following ADP-ribosylation by ExoS to a stoichiometry approaching 2 mol of ADP-ribose per mol of Ras was digested with trypsin and subjected to mass spectrometry. Mass spectra of peptides from ADP-ribosylated Ras possessed two unique peptides relative to peptides from non-ADP-ribosylated Ras. One peptide was identified which possessed a molecular mass of 3636 daltons, which corresponded to residues 17–42 of Ras plus 541 daltons. Addition of 541 daltons to each peptide corresponded to the void volume of the column (6 min), where NAD and free ADP-ribose eluted (data not shown). The two additional fractions that contained radioactivity eluted at 24 and 48 min, within the resolving region of the chromatogram.

The 24- and 48-min fractions were subjected to N-terminal amino acid sequencing. The first ten amino acids of the 24-min fraction contained a peptide corresponding to amino acids 38–47 of Ras, which included Arg⁴³ (Table I). Amino acid yields at each cycle were similar (within about 2-fold), with the exception of cycle 4 where there was a reduction in the yield of the predicted amino acid, Arg⁴¹. Others have demonstrated that ADP-ribosylarginine is hydrolyzed by treatment with strong base (5). During the coupling of the free N terminus with phenyl isothiocyanate, the peptide was subjected to pH 9.0. When the products of cycle 4 of the Edman degradation were analyzed, no aberrant peak corresponding to ADP-ribosylarginine or hydrolyzed ADP-ribosylarginine was detected, and only minute amounts of free arginine were detected. The absence of hydrolytic products of ADP-ribosylarginine indicated that the ADP-ribose—arginine bond was not cleaved during the Edman degradation procedure. Hydrophilic phenyl isothiocyanate-modified amino acids will not be extracted with butyl acetate from the glass fiber filter during Edman degradation as shown in the case of cysteine.² The fact that radioactivity was recovered on the glass fiber filter but not in the eluate of the Edman degradation is consistent with the idea that ADP-ribosylated arginine was not extracted from the glass fiber filter during cycle 4, and Arg¹ is one site of ADP-ribosylation. The first ten amino acids of the 48-min fraction contained a peptide corresponding to amino acids 127–136 of Ras, which included Arg¹²⁸ and Arg¹³⁵ (Table I). Amino acid yields at each cycle were similar (within about 2-fold), with the exception of cycle 2 where there was a reduction in the yield of the predicted amino acid, Arg¹²⁸. This was consistent with Arg¹²⁸ having been ADP-ribosylated. The high yield of Arg at cycle 9 indicated that Arg¹³⁵ had not been ADP-ribosylated and indicated that the reduction in yield seen in cycle 2 of the 48-min fraction and cycle 4 of the 24-min fraction was not because of a generalized decrease in yield of arginine after Edman degradation.

The sites that ExoS ADP-ribosylated on wild-type Ras were also determined by mass spectrometry. Ras alone or following ADP-ribosylation by ExoS to a stoichiometry approaching 2 mol of ADP-ribose per mol of Ras was digested with trypsin and subjected to mass spectrometry. Mass spectra of peptides from ADP-ribosylated Ras possessed two unique peptides relative to peptides from non-ADP-ribosylated Ras. One peptide was identified which possessed a molecular mass of 3636 daltons, which corresponded to residues 17–42 of Ras plus 541 daltons, while a second peptide was identified which possessed a molecular mass of 1914 daltons, corresponding to residues 124–135 of Ras plus 541 daltons. Addition of 541 daltons to each peptide was consistent with the addition of 1 ADP-ribose moiety to the peptide. These data supported the determination that ExoS ADP-ribosylates Ras at Arg⁴¹ and Arg⁴¹.

**Identification of Arg⁻¹⁵ as an Alternative Site of ADP-ribosylation of Ras by ExoS—** Earlier data indicated that ExoS ADP-ribosylated Ras at multiple sites and that Ras possessed undefined alternative sites of ADP-ribosylation (10). To identify a possible alternative site of ADP-ribosylation within Ras, a double Ras mutant, Ras₆CAAXR₄₁K,R₁₂₈K, was engineered by site-directed mutagenesis. Under saturation conditions, ExoS ADP-ribosylated Ras₆CAAXR₄₁K,R₁₂₈K to a stoichiometry of approximately 1 mol of ADP-ribose/mol of Ras (Table II). This was consistent with the presence of an alternative site of

² L. Mende-Mueller, personal communication.
ADP-ribosylation within the double mutant. Under linear velocity conditions, ExoS ADP-ribosylated wild type RasΔCAAX at a 4-fold greater velocity than RasΔCAAX-R41K,R128K (Fig. 2). These data suggested the alternative site of ADP-ribosylation was not accessible for ADP-ribosylation upon the ADP-ribosylation of Arg128, possibly because of steric limitations. The identity of the alternative site of ADP-ribosylation within RasΔCAAXR41K,R128K was determined as described for wild type RasΔCAAX. rpHPLC analysis of peptides from V8 protease-digested ADP-ribosylated RasΔCAAXR41K,R128K showed the elution of two major fractions of radioactivity (Fig. 1B). The first fraction containing radioactivity eluted with the void volume of the column (6 min), corresponding to NAD or hydrolyzed ADP-ribose, whereas the second fraction containing radioactivity eluted at 46 min. N-terminal amino acid sequencing of the first eleven amino acids of the 46-min fraction iden-

TABLE I

N-terminal amino acid sequencing of Ras peptides that have been ADP-ribosylated by ExoS

Peptide sequencing was performed with an ABI371 automated sequencer with the preparation of peptides as described under "Experimental Procedures."

| Cycle | Wild type Ras (24-min peptide) | Wild type Ras (48-min peptide) | RasR41K,R128K (46-min peptide) |
|-------|--------------------------------|--------------------------------|--------------------------------|
|       | Amino acid | Recovery | Amino acid | Recovery | Amino acid | Recovery |
| 1. Asp | 19.6 pmol | Ser | 50.5 pmol | Ser | 26.9 pmol |
| 2. Ser | 6.9 pmol | Arg | 6.5 pmol | Lys | 46.6 pmol |
| 3. Try | 11.7 pmol | Gln | 93.3 pmol | Gln | 36.9 pmol |
| 4. Arg | 1.6 pmol | Ala | 84.8 pmol | Ala | 39.0 pmol |
| 5. Lys | 14.4 pmol | Gln | 80.2 pmol | Gln | 28.9 pmol |
| 6. Gln | 13.1 pmol | Asp | 48.9 pmol | Asp | 25.9 pmol |
| 7. Ala | 11.2 pmol | Leu | 83.1 pmol | Leu | 24.6 pmol |
| 8. Val | 11.2 pmol | Ala | 76.3 pmol | Ala | 22.4 pmol |
| 9. Ile | 8.7 pmol | Arg | 47.1 pmol | Arg | 1.3 pmol |
| 10. Asp | 7.5 pmol | Ser | 21.4 pmol | Ser | 6.0 pmol |
| 11. Tyr | 15.7 pmol | | | | |
Identification of Primary Amino Acid Homology between the Catalytic Domain of ExoS and Vertebrate ADP-ribosyltransferases—To date, several vertebrate ADP-ribosyltransferases have been identified, including rabbit skeletal muscle ADP-ribosyltransferase (RNART) (19), rat RT6 (20), a human ecto-ADP-ribosyltransferases (21), and chicken ADP-ribosyltransferase types I and II (22). Several of the vertebrate ADP-ribosyltransferases have the capacity to ADP-ribosylate several eukaryotic target proteins (11, 12) and to ADP-ribosylate at two independent sites (13, 14), which identified functional relationships with ExoS. Using the tFASTA algorithm, the vertebrate ADP-ribosyltransferases were observed to possess considerable primary amino acid homology with the catalytic portion of ExoS. ExoS possessed homologies of 26.6% with RNART, 28.3% with HNART, 31.9% with RT6, 35.4% with RNART, and 38.1% with HNART.

Influence of Nucleotide Occupancy on the Ability of ExoS to ADP-ribosylate Ras—Exoenzyme S ADP-ribosylates Ras at two distinct locations. The structure of GDP-bound Ras residues 1–169 (Protein Data Bank code 4Q2I; Ref. 18) was visualized, and the sites of ADP-ribosylation were labeled using Bobscript software. One site of ADP-ribosylation, arginine 41 (R41), is in a b-sheet which is adjacent to the switch 1 domain of Ras, whereas the second and alternative sites of ADP-ribosylation, arginine 128 (R128) and arginine 135 (R135), respectively, are in an a-helix near the C terminus.

**ADP-ribosylation of Ras and Ras mutants by Exoenzyme S**

Wild type Ras, RasCAAX, or RasCAAXR41K,R128K were prepared as described under “Experimental Procedures.” For loading experiments, 20 μM Ras was incubated at 30 °C in 200 mM Na acetate, containing 1 μM GTP-S or GDP. The reaction was stopped by the addition of 20 mM MgCl2 after 30 min. Velocities of ADP-ribosylation of wild type Ras were determined in reaction mixtures containing 5 μM Ras and 1.2 nM ΔN222 ExoS. Stoichiometries of the ADP-ribosylation of wild type Ras was determined in reaction mixtures containing 5 μM Ras and 12 nM ΔN222 ExoS. Stoichiometry of the ADP-ribosylation of RasCAAX (wild type or mutant) was determined in reaction mixtures containing 5 μM Ras and 4 nM ΔN222 ExoS.

|wild type Ras| Wild type Ras loaded with GTP| Wild type Ras loaded with GDP| Wild type Ras loaded with Mg| RasCAAX| RasCAAX R41K,R128K|
|---|---|---|---|---|---|
|mol ADP-ribose/ mol Ras| 2.08 ± .21| 2.08 ± .33| 2.21 ± .16| 1.94 ± .10| 2.03 ± .14| 1.33 ± .04|
|mol ADP-ribose/min/ mol ΔN222| 144 ± 6| 112 ± 18| 125 ± 16| 135 ± 25| 92 ± 16| 22 ± 10|

* Represents the results of two experiments performed in duplicate.

**Fig. 2.** ExoS ADP-ribosylates R41K, R128K RasCAAX at a lower rate than wild type RasCAAX. Reaction mixtures contained: 200 mM Na acetate (pH 6.0), 50 μM [adenylate phosphate-32P]NAD, 0.4 nM ΔN222 ExoS, 5 μM Ras, and 4 nM FAS. Reactions were stopped at the indicated times by spotting the reaction mixture on trichloroacetic acid paper. Data represent the results of three experiments performed in duplicate.

**Fig. 3.** Exoenzyme S ADP-ribosylates Ras at two distinct locations. The structure of GDP-bound Ras residues 1–169 (Protein Data Bank code 4Q2I; Ref. 18) was visualized, and the sites of ADP-ribosylation were labeled using Bobscript software. One site of ADP-ribosylation, arginine 41 (R41), is in a b-sheet which is adjacent to the switch 1 domain of Ras, whereas the second and alternative sites of ADP-ribosylation, arginine 128 (R128) and arginine 135 (R135), respectively, are in an a-helix near the C terminus.
FIG. 4. Alignment of P. aeruginosa exoenzyme S with eukaryotic ADP-ribosyltransferases. A portion of the catalytic domain of Exoenzyme S was aligned with the eukaryotic ADP-ribosyltransferases, using Pileup from GCG. **RNA**T is the rabbit skeletal muscle NAD ADP-ribosyltransferase, **RNA**T is the human NAD ADP-ribosyltransferase, **CHA**T 1 is the chicken ADP-ribosyltransferase type 1, **CHA**T 2 is the chicken ADP-ribosyltransferase type 2, and **RT**6 is the rat ADP-ribosyltransferase. Residues conserved between the eukaryotic transferases and Exoenzyme S are shown in **bold**. Region 2 of the ADP-ribosyltransferases corresponds to the STS sequence, whereas region 3 of the transferases corresponds to the catalytic glutamic acids.

**DISCUSSION**

Previous data indicated that exoenzyme S ADP-ribosylated Ras at multiple sites, including Arg41 and a second site which could not be localized. The current study utilized direct biochemical and biophysical approaches to analyze how ExoS modified Ras. ADP-ribosylated Ras was digested with S. aureus V8 protease, and radiolabeled peptides were subjected to Edman degradation. During the Edman degradation reaction, radiolabeled ADP-ribosylated arginine was not recovered although a decrease in the yield of arginine at residues corresponding to Arg41 and Arg128 of Ras was detected. Others have demonstrated that radiolabeled ADP-ribosylarginine was not recovered when peptides ADP-ribosylated at arginine are subjected to Edman degradation although the site of ADP-ribosylation can be identified indirectly by a decrease in amino acid yield at a candidate arginine residue (14). The fact that arginines 41 and 128 were identified as the sites of ADP-ribosylation suggests that ExoS has a limited substrate specificity for arginines 41 and 128 of Ras, consistent with a steric mechanism of blockage of ADP-ribosylation.

The plasticity of the ExoS-Ras interactions may explain the ability of ExoS to ADP-ribosylate numerous small molecular weight GTP binding proteins in vitro (7). Coburn et al. (7) showed that ExoS ADP-ribosylates several members of the Ras superfamily in vitro. Only a limited subset of the family, Ras, Ral, and Rap, contained the Arg41 homologue. Alignment of the α-helix, which contains the second site ADP-ribosylation, identified numerous members of the Ras superfamily that contained an arginine residue at the Arg41 homologue as a potential site for ADP-ribosylation. However, no distinct recognition motif is apparent from examination of the primary amino acid sequences of members of the Ras superfamily (Fig. 5). Consistent with this prediction, we have observed that ExoS can ADP-ribosylate numerous small molecular weight GTP binding proteins in vitro (7). RhoA does not contain an Arg41 homolog but does contain several Arg residues within the secondary α-helix. The potential of ADP-ribosylating Ras superfamily members at their Arg41 or Arg128 homologues may have different functional implications. Arg41 is a contact residue in the Ras-Rap co-crystal structure (23) and is in a region of close contact in the Ras-SOS crystal structure (24). Modification of Ras at Arg41 may inhibit Ras activation or Ras-Raf interactions. The secondary site helix has no ascribed function, although it is located adjacent to the Ras-SOS crystal site. Therefore, it is unclear how ADP-ribosylation of Ras at Arg128 or 135 would affect Ras function, or alternatively ADP-ribosylation could affect Ras posttranslational modification. While ExoS has been shown to ADP-ribosylate Ras in vivo (9), a more detailed analysis of the in vivo targets of ExoS is needed.

Neither nucleotide occupancy nor Mg2+ binding altered the
ability of ExoS to ADP-ribosylate Ras, as the velocity and stoichiometry of the ADP-ribosylation of GTP-loaded, GDP-loaded, and nucleotide-free Ras were essentially identical. This indicated that the sites of ADP-ribosylation were not involved in nucleotide binding or chelation of Mg$^{2+}$ and was consistent with structural data which predicted that Arg$^{41}$ and Arg$^{128}$ were distant from these binding sites. In contrast to ExoS, several bacterial toxins that modify Ras superfamily members preferentially target GDP bound forms of those proteins; *Clostridium sordellii* LT preferentially glucosylates GDP bound Ras (25) and *Clostridium botulinum* C3 ADP-ribosyltransferase preferentially ADP-ribosylates GDP-bound Rho (26). The fact that ExoS modifies both the GTP- and GDP-bound forms of Ras whereas other toxins described to date modify only the GDP-bound form indicates that ExoS modifies Ras signal transduction by a mechanism that is distinct from the Cloridial toxins.

The endogenous vertebrate ADP-ribosyltransferases share several properties with ExoS, including the ability to ADP-ribosylate multiple target proteins and the ability to ADP-ribosylate target proteins at multiple sites. Sequence alignment showed that the catalytic domain of ExoS aligns more extensively with the eukaryotic ADP-ribosyltransferases than bacterial ADP-ribosyltransferases. The primary amino acid alignments of mono-ADP-ribosyltransferases predicts the conservation of a basic amino acid, a Ser-Thr-Ser sequence, and a catalytic glutamic acid (27). Alignment between the catalytic domain of ExoS and the vertebrate ADP-ribosyltransferases extended from the Ser-Thr-Ser sequence through the active site glutamic acid. In contrast, tFasta alignment between the catalytic domain of ExoS and the bacterial ADP-ribosyltransferases, CT, LT, and PT, identified alignments only within the Ser-Thr-Ser sequence. This suggests that with respect to both functional and sequence alignments, ExoS is more similar to the vertebrate ADP-ribosyltransferases than the prokaryotic ADP-ribosyltransferases. Thus, ExoS may have an evolutionary link with the vertebrate ADP-ribosyltransferases. A better understanding of the mechanism by which ExoS modifies eukaryotic physiology may provide insight into the mechanisms of action of the vertebrate ADP-ribosyltransferases.

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