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Natural transformation as a mechanism of horizontal gene transfer among environmental *Aeromonas* species☆

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ABSTRACT

Aeromonas species are common inhabitants of aquatic environments and relevant as human pathogens. Their potential as pathogens may be related in part to lateral transfer of genes associated with toxin production, biofilm formation, antibiotic resistance, and other virulence determinants. Natural transformation has not been characterized in aeromonads. DNA from wild-type, prototrophic strains that had been isolated from environmental sources was used as donor DNA in transformation assays with auxotrophs as the recipients. Competence was induced in 20% nutrient broth during the stationary phase of growth. Optimal transformation assay conditions for one chosen isolate were in Tris buffer with magnesium or calcium, pH 5–8, and a saturating concentration of 0.5 μg of DNA per assay (3.3 ng of DNA μl^{-1}) at 30 °C. Sodium was also required and could not be replaced with ammonium, potassium, or lithium. The maximal transformation frequency observed was 1.95×10^{-3} transformants (recipient cell)$^{-1}$. A survey of environmental *Aeromonas* auxotrophic recipients ($n = 37$), assayed with donor DNA from other wild-type environmental aeromonads under optimal assay conditions, demonstrated that 73% were able to act as recipients, and 100% were able to act as donors to at least some other aeromonads. Three different transformation groups were identified based on each isolates' ability to transform other strains with its DNA. The transformation groups roughly corresponded to phylogenetic groups. These results demonstrate that natural transformation is a general property of *Aeromonas* environmental isolates with implications for the genetic structures of coincident *Aeromonas* populations.

Introduction

Aeromonads are implicated in self-limiting gastrointestinal infections [3,4,24] and extraintestinal infections, with wounds serving as the usual portal of entry [20]. Both environmental and clinical aeromonad isolates demonstrate resistance to antimicrobial agents, making the treatment of infections more difficult [23,55]. These resistances can be transferred by plasmids [22,41] or through the process of natural transformation. Evolutionarily recent high frequency of horizontal gene transfer among aeromonads has been reported [42]. The mechanism of these gene transfer events is unknown. Approximately 90 prokaryotic species have been characterized as naturally transformable [14] occurring in diverse ecological groups, such as photolithotrophs, chemolithotrophs, heterotrophs, methylotrophs, clinical pathogens, and members of *Archaea* [25].

Natural transformation in *Aeromonas* is not widely known or studied. Sakai [39,40] reported that a single strain of *A. salmonicida* was capable of competence and transformation but gave no detailed descriptions of the mechanisms involved or the conditions under which transformation occurred. No research has characterized this process in any aeromonad, nor have the evolutionary or clinical consequences of transformation in these ubiquitous aquatic organisms been considered. The goals of this study were to determine if *Aeromonas* species isolated from streams and lakes are generally competent for natural transformation, characterize optimal conditions for transformation in the laboratory, determine if the recognized species of aeromonads can be induced to competence under the same conditions, and assess the scope and evolutionary patterns of transformability within the genus.

Aeromonas isolates have been categorized into 30 validly published species names to date [5,6,15,19,29]. A limited number of biochemical tests can be used to place most strains into one of three phenotypic complexes [1]. The *A. hydrophila* complex is comprised of *A. hydrophila*, *A. bestiarum*, and *A. salmonicida*. The *A. caviae* complex is comprised of *A. caviae*, *A. media*, and *A. eucrenophila*. The *A.*

☆ The GenBank accession numbers for the sequences reported in this paper are GenBank: FJ159389 to FJ159424.

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sobria complex is comprised of *A. veronii* biovar sobria, *A. jandaei*, *A. schubertii*, and *A. trota* [1].

Materials and methods

Bacterial strains and identifications with gyrB sequences

Aeromonas strains used in this investigation were isolated from rivers and playa lakes in west Texas and New Mexico [18]. DNA was extracted and purified from each strain using the method of Ulrich and Hughes [51]. The *gyrB* gene sequences, using the same primers and conditions as described by Yañez et al. [57], were amplified in order to obtain species identifications of the isolates and reconstruct a phylogenetic history. The 1100-bp *gyrB* PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, Oh.) to remove primers and extra dNTPs and sequenced at the Core Facility of the Texas Tech University Center for Biotechnology and Genomics. Sequences were analyzed using Vector NTI, 9.0.0 (Invitrogen, Carlsbad, Ca.), deposited into GenBank (accessions FJ159389 to FJ159424), and were compared against all microbe sequences deposited in NCBI using BLAST [7]. Two aeromonads from the American Type Culture Collection (ATCC), *A. caviae* ATCC 15468^T and *A. veronii* biovar sobria ATCC 9071, were used to confirm the accuracy of the sequences and identifications.

Mutagenesis and screening of aeromonads

Thirty-five wild-type environmental aeromonads and the two *Aeromonas* ATCC strains were mutagenized to auxotrophy with diethyl sulfate (DES) (Sigma–Aldrich, St. Louis, MO). Each aeromonad strain was inoculated into 2 ml of no citrate E (NCE) minimal medium [9] supplemented with 0.25% glucose (Sigma) and incubated with aeration overnight at 30 °C. Four drops of DES were added to 5 ml of each culture diluted 1:10 in NCE buffer, and the tubes incubated at room temperature for 1 h. Then, 0.1 ml of the DES-treated cells was inoculated into 2 ml of Difco tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD). The cells were incubated with aeration at 30 °C overnight, and 0.1 ml was inoculated into 2 ml of NCE glucose minimal medium and incubated for 2 h at 30 °C. Cefuroxime (Sigma) was added for a final concentration of 30 μg ml^{-1} to counter select for non-growing, auxotrophic cells. The cells were then incubated at 30 °C for 3 h. The cultures were diluted in NCE buffer, spread onto Difco tryptic soy agar (TSA) (Becton Dickinson), and incubated overnight. Colonies were patched to TSA, incubated overnight, and replica-plated to NCE glucose minimal agar. Auxotrophs that did not grow on minimal medium were subcultured and used in later experiments.

Quantitative transformation assays

Competent cells were prepared by inoculating a single colony into 20% nutrient broth (NB) (Becton Dickinson), pH 7.5, and incubating with aeration overnight at 30 °C. Each culture was diluted 1:100 in 20% NB and incubated again with aeration for 24 ± 2 h at 30 °C. The competency protocol was derived through trial and error in preliminary experiments that sought to obtain maximum response. Quantitative transformation assays were performed in 1.5-ml microcentrifuge tubes that contained 100 μl of standard transformation buffer consisting of 53.5 m*M* Tris (Sigma), pH 7.9, 20 m*M* $MgSO_4$ (Spectrum Chemical, Gardena, CA), and 50 m*M* NaCl (EM Science, Gibbstown, NJ). Competent cells were added in a volume of 40 μl [$(4.0 \pm 1.5) \times 10^7$ colony-forming units (CFU)]. Pure DNA from each wild-type aeromonad was prepared according to the method of Ulrich and Hughes [51]. One microgram of DNA was added in a volume of 10 μl (final concentration of 6.7 ng μl^{-1}). The assay mixture was incubated at 30 °C for 30 min. After the initial incubation, transformation was terminated by adding 10 μl of 200 μg ml^{-1} of deoxyribonuclease I (DNase I) (Sigma) solution and then incubating at 30 °C for 1 h to degrade free DNA. Controls were performed by adding DNase I to the assay mixture before the initial 30-min incubation to eliminate transformation. No-DNA and no-cell assays were also performed as negative controls; no transformants ever appeared on any of the control plates. Assay mixtures were diluted in 0.85% NaCl, spread onto NCE glucose minimal agar, and incubated 48 h at 30 °C. Colonies that grew on NCE glucose minimal agar were considered to be transformants. Transformants were counted, and transformation frequencies were determined by dividing the number of transformants by the initial number of CFUs added.

Determining optimal competence induction medium

One auxotroph (C-70, a histidine mutant derived from putative *A. salmonicida* subsp. *pectinolytica* strain 92 originally isolated from the North Fork of the Brazos River) was chosen for its high level of transformability to characterize optimal competence induction and transformation conditions. A single colony was inoculated into 20% NB and incubated with aeration overnight at 30 °C. The culture was diluted 1:100 in 20%, 40%, 60%, 80%, and 100% NB; 20% and 100% Luria broth (LB); 20% and 100% TSB; and NCE glucose minimal broth supplemented with 0.1 m*M* L-histidine (Sigma) and incubated with aeration overnight at 30 °C. Transformation assays were performed in triplicate in standard transformation buffer as described above. Transformation frequencies were calculated as "transformants per recipient" and used as an indicator of competence.

Effect of growth phase on competence

One colony of *Aeromonas* C-70 was inoculated into 2 ml of 20% NB, pH 7.5, and incubated with aeration overnight at 30 °C. The 2-ml culture was inoculated into 200 ml of 20% NB in a flask and incubated at 30 °C with aeration. Samples were taken hourly for 24 h and then at 36 h and 48 h. The absorbance of each sample was measured at a wavelength of 540 nm using a Spectronic Genesys 5 spectrophotometer (Spectronic Instruments, Rochester, NY). Samples were diluted in 0.85% NaCl and plated onto 20% Difco nutrient agar (Becton Dickinson), pH 7.5, and incubated at 30 °C overnight for viable counts. Samples were assayed for transformation as described above. This procedure was repeated with three different flasks and two replicates per flask at each sampling time.

Characterization of optimal transformation conditions

Assays for determining optimal transformation conditions were performed as described above for quantitative transformation assays except that each parameter was varied independently. Preliminary experiments indicated that NaCl and $MgSO_4$ were required for transformation to occur. Concentrations of NaCl and $MgSO_4$ were varied to determine optimal concentrations of each to maximize transformation. Varying concentrations of KCl (Fisher Scientific, Pittsburgh, PA), LiCl (Sigma), and NH_4Cl (Fisher Scientific) were tested in place of NaCl. Varying concentrations of $CaCl_2$ (Mallinckrodt Baker, Phillipsburg, NJ) were tested in place of $MgSO_4$. Other variations included Tris concentration, temperature, pH, DNA concentration, and DNA incubation time with cells before adding DNase I. Assays were performed on different days in triplicate or quadruplicate.

Scope of transformability

Quick transformation assays were performed in order to determine the scope of transformability among diverse aeromonad strains isolated from different aquatic sources. This experiment was intended to give a broad overview of transformability of environmental aeromonads, not a quantitative analysis. Six of the 37 assays were repeated in triplicate on different days. Crude DNA from the wild-type (prototrophic) strain was prepared using the procedure of Juni [21]. Briefly, a pellet of each bacterial culture was suspended in lysis buffer and incubated at 70 °C for 1 h, killing cells and inactivating any bacteriophages that might be present, and yielding approximately 1 μg μl^{-1} of crude DNA. Each auxotrophic strain was tested for transformation of DNA from all of the other wild-type strains. Assay buffer (50 μl) was added to 1.5-ml microcentrifuge tubes together with 20 μl of competent cells and 10 μl of crude DNA. Each assay was incubated at 30 °C for 1 h, and 20 μl were inoculated as a grid on NCE glucose minimal agar without DNase I treatment. The plates were incubated at 30 °C for 48 h, and numbers of transformants were counted. If assays yielded uncountable colonies, then 50 colonies were used in the statistical analyses. Cluster analysis based on Ward's algorithm [54] was performed in PAST v2.17 [16] in order to identify transformation groups based on each isolate's ability to receive and donate DNA from each of the other isolates, compared in a pairwise matrix. The degree to which the resulting dendrograms reconstructed the original data matrix was measured as the cophenetic correlation coefficient [37], on a scale from 0 (no agreement) to 1 (complete agreement). Bootstrap support for groupings was determined by 1000 resampling replicates. Further, a heatmap of transformation groups based on Ward's clustering was created using the default settings of "heatmap.2" in the "gplots" package of R [36].

Phylogenetic data analysis

The *gyrB* nucleotide sequences from all *Aeromonas* strains in this study and the top BLAST hits from GenBank were aligned using the MEGA v4.0.2 [41] interface to ClustalW [50], with a gap opening penalty of 15 and a gap extension penalty of 6.6 [30]. A neighbor-joining (NJ) tree [15] was estimated with MEGA v4.0.2 using all codon positions and complete deletion of gaps. Pairwise distances were computed using the maximum composite likelihood method [49] and are in units of number of base substitutions per site. Bootstrap support values were estimated using 1000 pseudoreplicate searches [13]. Maximum parsimony (MP) analyses were carried out using PAUP* v4.0b10 [48]. Gaps were treated as missing data. Data were analyzed using heuristic searches with random taxon addition and tree-bisection-reconnection (TBR) branch swapping, saving one tree per replicate; 1000 replicated searches were conducted to search for islands of equally most parsimonious trees. Bootstrap support values were estimated by 1000 MP bootstrap searches using the heuristic search procedures above but with a maximum tree limit of 100 for each random taxon addition replicate. Maximum likelihood (ML) analyses were performed using the RAxML program [47] as implemented through the CIPRES web portal (http://www.phylo.org/). Gaps were treated as missing data, and each codon position was treated as a separate partition, allowing GTR model parameters, branch lengths, and alpha parameter of the gamma distribution to be estimated independently for each codon position. The ML tree was chosen from the combined results of 1000 replicated searches. Bootstrap support values were estimated by 1000 ML bootstrap searches using search procedures above. Trees were rooted using *A. schubertii* as an out-group because *A. schubertii* is the most genetically distinct species, and preliminary ML analyses (not shown) with *gyrB* sequences from *Ferrimonas balearica* (GenBank: AB193759.1) and *Listonella anguillarum* (GenBank: JQ698507.1) as outgroups suggested that *A. schubertii* is sister to a clade containing the rest of the *Aeromonas* species included in this study.

Due to the potential of recombinant DNA in strains undergoing transformation, all *gyrB* sequences were extensively inspected for chimeras using RDP3 [28] following the approach of Brokaw and Hufford [11]. Briefly, maximum parsimony analyses and fast bootstraps were performed using PAUP* to detect signs of homoplasy caused by recombination as either long terminal branches or long internal branches with low bootstrap support. Searches for chimeras were carried out using all recombination detection methods implemented in RDP3 by systematically increasing *P*-value cutoffs for each method, resulting in numerous potential recombinants with biases favoring false positives. To exclude false positives, we ran MP analyses using segments of the putative false positives separated at the recombination points (Fig. S1) suggested by RDP3. If these segments were not placed in conflicting positions in the resulting topologies, then the original sequences were assumed to be nonrecombinant and were used in all subsequent phylogenetic analyses. Because only partial *gyrB* sequences were available for some strains due to recombination, MP and ML analyses were performed with separate partial recombinant sequences divided at recombination points and with recombinant sequences excluded to determine the effects of missing data on phylogenetic resolution and clade support. In a few cases, very short partial sequences were excluded from all analyses because too few parsimony informative characters were available to reconstruct relationships.

To examine whether differences in isolates' ability to receive and donate DNA were related to phylogenetic proximity, we constructed distance matrices representing transformation function and matrices of phylogenetic distances in Mesquite v2.75 [26] and conducted Mantel tests [17,46] in PAST [16]. Two separate matrices representing transformation function were constructed containing the Euclidean distances between isolates based on abilities (1) to receive DNA (transformability) and (2) to donate DNA from of each of the 35 isolates and the two ATCC strains ("donatability"). A phylogenetic distance matrix was based on the branch lengths separating species (patristic distances) on the ML tree. When isolates were represented by chimeric *gyrB* sequences, the matrix was based on the phylogenetic positions of the longest segments from the divided sequences. The pairwise distances from a NJ tree based on DNA sequences without chimeras separated were used as a second approximation of phylogentic distance. Mantel tests were performed comparing each combination of functional and phylogenetic distances. The *P*-values in Mantel tests were based on a distribution of *r*-values created from 10,000 random pairwise permutations and comparison of the observed *r*-values to this distribution.

The results of the Ward's cluster analyses suggested that our quantitative measurements of transformability depend on at least two variables, DNA uptake ability and DNA preference. Because the Ward's cluster analysis for transformability was found to separate groups based primarily on uptake ability and not DNA preference, we performed an additional analysis to test for phylogenetic signal in DNA preference alone. Aeromonad strains were assigned discrete character states based on their preferences for DNA from specific "donatability" groups designated by the Ward's cluster analysis for "donatability" (Table S1). Strains that exhibited no transformation were coded as "0." Competent strains were coded "1," "2," or "3" (treated as discrete, unordered characters) based on the "donatability" group that they preferred. We tested for significant phylogenetic signal in DNA preference by transformers using Mesquite following the approach of Maddison and Slatkin [27]. This method tested whether related species have more similar preferences than expected by chance, and is based on the number of

Table 1
Effect of different culture media on competence induction in strain C-70 as measured by transformation frequency at 24 h post-inoculation (mean ± SE). NB, nutrient broth; LB, Luria broth; TSB, tryptic soy broth; GMB + his, NCE glucose minimal broth supplemented with 1 m*M* histidine. Transformation frequencies were determined by dividing the number of transformants by the initial number of cells added per assay (4.0×10^7 cells). A transformation frequency of 8.33×10^{-9} transformants (recipient cell)$^{-1}$ indicates that one transformant appeared on one of the three replicate assay plates. This is the lowest transformation frequency that can be measured using this protocol.

Media	Transformants (recipient cell)$^{-1}$
20% NB	$(3.38 \pm 0.20) \times 10^{-4}$
40% NB	$(1.32 \pm 0.12) \times 10^{-5}$
60% NB	$(3.33 \pm 3.12) \times 10^{-8}$
80% NB	$<8.33 \times 10^{-9}$
100% NB	$<8.33 \times 10^{-9}$
20% LB	8.33×10^{-9}
100% LB	$<8.33 \times 10^{-9}$
20% TSB	8.33×10^{-9}
100% TSB	8.33×10^{-9}
20% GMB + his	$<8.33 \times 10^{-9}$

character state changes reconstructed in the tree using parsimony. *P*-values representing the probability that the observed number of changes in DNA preference occurred by chance (i.e. not phylogenetically constrained) were determined by comparison to a null model in which data were reshuffled 1000 times across the tips of the phylogeny.

Results

Identification of competent aeromonad

C-70, a histidine auxotrophic mutant, was obtained through DES mutagenesis of a prototrophic strain, *Aeromonas* sp. strain 92, isolated from sediment from the north fork of the Brazos River [18]. This wild-type strain was tentatively identified as a species of *A. salmonicida* subsp. *pectinolytica* based on *gyrB* sequence homology to GenBank accession AM262158. This subspecies is characterized by growth at both 30 °C and 37 °C [34]. Preliminary results showed that C-70 was easily transformable and does not spontaneously revert to prototrophy at a detectable frequency [$<2.50 \times 10^{-8}$ revertants (recipient cell)$^{-1}$]. C-70 was used for optimization of competence induction and transformation conditions.

Competence induction conditions

Competence in *Aeromonas* C-70 was inducible. Cells grown in 20% NB yielded the greatest transformation frequencies. Full-strength standard growth media, both complex and defined, inhibited transformation (Table 1). Competence began to appear during stationary phase at about 12 h after inoculation into fresh medium (Fig. 1) and was maintained through at least 48 h. Maximal competence occurred in late stationary phase at 22–24 h after inoculation.

Optimal transformation conditions

Transformation assays without NaCl did not yield any transformants, indicating that sodium is required. Maximal transformation frequencies occurred with 33 m*M* NaCl (Fig. 2a). Other monovalent cations, K^+, Li^+, and NH_4^+, could not replace Na^+ (Fig. 2a) and yielded transformation frequencies that were barely detectable [$0–3.9 \times 10^{-5}$ transformants (recipient cell)$^{-1}$]. Transformation was also dependent on either magnesium or calcium. Maximal transformation frequencies occurred with 17 m*M* $MgSO_4$ [8.5×10^{-4} transformants (recipient cell)$^{-1}$] or with 17 m*M* $CaCl_2$ [1.4×10^{-3} transformants (recipient cell)$^{-1}$] (Fig. 2b). Magnesium

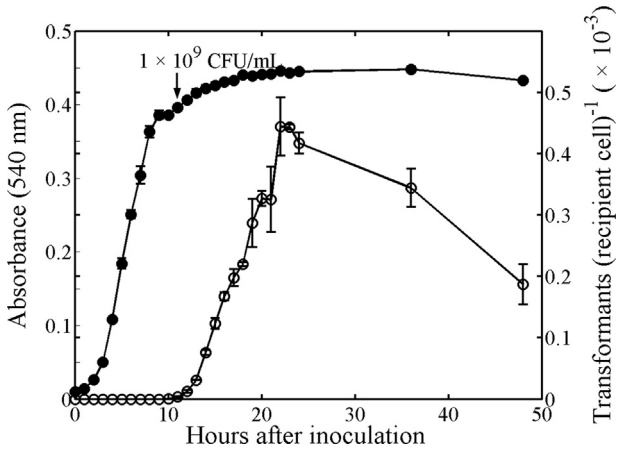

Fig. 1. Effect of growth phase on competence induction. Symbols: (○) optical density at 540 nm, (●) transformation frequency, (↓) beginning of stationary phase (equivalent to 1×10^9 CFU ml^{-1}), ($n = 3$). Error bars indicate ±1 SE and are a result of three independent cultures with two replicates each.

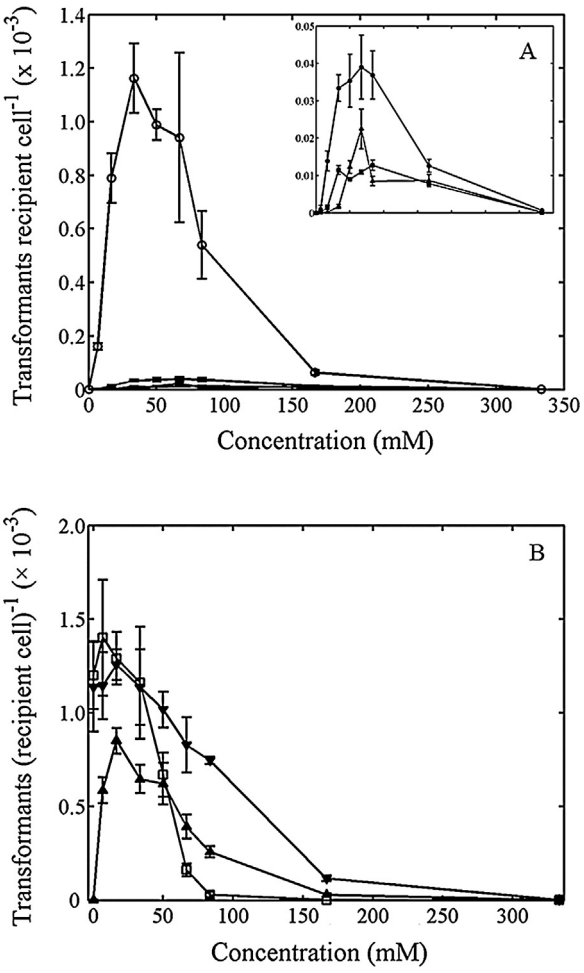

Fig. 2. Effect of cations and buffer on transformation. (a) Effect of monovalent cation concentrations on transformation frequency. Symbols: (○), varying NaCl + 13 m*M* $MgSO_4$, (●) varying KCl + 13 m*M* $MgSO_4$, (■) varying LiCl + 13 m*M* $MgSO_4$, (△) varying NH_4Cl + 13 m*M* $MgSO_4$. (b) Effect of divalent cation and buffer concentrations on transformation frequency. Symbols: (□) Varying $CaCl_2$ + 33 m*M* NaCl, (▲), varying $MgSO_4$ + 33 m*M* NaCl, (▼) varying Tris + 33 m*M* NaCl + 13 m*M* $MgSO_4$. Values are means ($n = 4$) ±1 SE and are a result of three independent assays with three plates each.

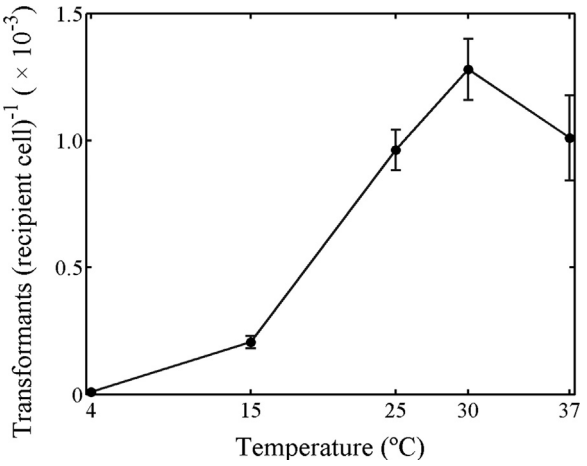

Fig. 3. Effect of temperature on transformation frequency. Values are means ($n = 4$) ±1 SE.

was added to subsequent transformation assays instead of calcium so as to ensure that natural transformation was occurring as opposed to chemical (artificial) transformation that is usually performed with calcium chloride. Tris concentration had less influence on transformation frequency than sodium and divalent cations did (Fig. 2b). Preliminary experiments indicated that higher transformation frequencies occurred in Tris buffer than in phosphate (NCE) buffer. Sterile distilled water with added sodium and magnesium yielded transformants. Strong transformation occurred from 0 to about 50 m*M* Tris, pH 8.0, and decreased with higher concentrations.

The optimal temperature for transformation was 30 °C [$(1.28 \pm 0.12) \times 10^{-3}$ transformants (recipient cell)$^{-1}$] (Fig. 3). Transformation frequency increased with increasing temperature from 15 to 30 °C and decreased at 37 °C (Fig. 3). Buffer pH had only a small effect on aeromonad transformation in the pH range of 5–9. Transformation frequencies varied between 0.97×10^{-3} (at pH 9) and 1.8×10^{-3} (at pH 5) transformants (recipient cell)$^{-1}$ (data not shown). There was no significant difference in the mean frequencies obtained from assays with pH 5, 6, 7, and 8. Transformation frequencies for assays with pH 9 were significantly lower than those from pH 5–8. Subsequently, the quantitative assay protocol was always performed at pH 7.9. Transformation frequency was greatest with a DNA concentration of 0.5 μg of DNA per assay and did not increase with DNA concentration up to 4 μg of DNA per assay (Fig. 4a). The longer the time that DNA was incubated with competent cells before adding DNase I, the greater the transformation frequency [$(4.90 \pm 0.49) \times 10^{-4}$ transformants (recipient cell)$^{-1}$ at 240 min], although multiple pairwise comparisons showed that there was no significant difference in the means obtained from incubating 120 to 300 min (comparison data not shown) (Fig. 4b). Although our results show that maximum transformation frequency occurs after 120 min of incubation with DNA, we chose to limit our subsequent assays to 30 min to ensure that no cell division of transformants was occurring in the transformation assay that could possibly skew the resulting transformation frequencies and to also prevent death of transformants in the buffer due to prolonged exposure. There was an approximate 10-fold variability in the competence of C-70 between experiments performed on different days, so all results were compared to a control run within each experiment. The means of the transformation positive controls using the optimal conditions for the quantitative transformation assays varied from 0.138×10^{-3} to 1.33×10^{-3} transformants (recipient cell)$^{-1}$ ($n = 20$). The reversion frequency of the histidine mutant to prototrophy was below the detection

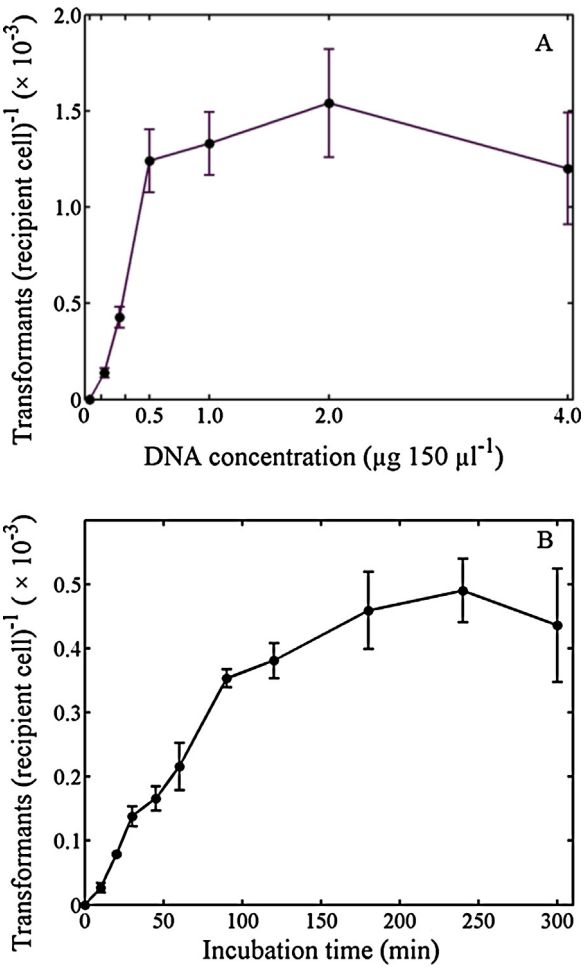

Fig. 4. The effects of DNA concentration and exposure time on transformation frequency. (a) Effect of DNA concentration. (b) Effect of DNA incubation time prior to DNase I addition. Competent cells were incubated with varying concentrations of DNA for 30 min, DNase I was added, and the assays were incubated for an additional 60 min before plating. (b) Effect of DNA incubation time prior to DNase I addition. Competent cells were incubated with 1 μg of DNA for varying time durations until DNase I was added, and the assays were incubated for an additional 60 min before plating. Values are means ($n = 3$) ±1 SE and are a result of three independent assays with three plates each.

limit of 2.50×10^{-8} revertants (recipient cell)$^{-1}$, since no revertants were detected in any of the no-DNA control assays.

Scope of transformability

Thirty-seven environmental aeromonad strains were mutagenized to auxotrophy with DES and evaluated. All reversion frequencies were too low to be detected under the test conditions [$<2.50 \times 10^{-8}$ revertants (recipient cell)$^{-1}$]. Quick, semi-quantitative transformation assays were performed in order to determine if all auxotrophic aeromonad strains could take up DNA from wild-type aeromonad strains under the test conditions. The assays were intended to give a broad overview of the scope of transformability in the total population rather than make precise measurements of transformation frequencies of individual strains. Nevertheless, these assays revealed substantial variation in transformation capabilities among the 37 strains (Fig. 5). Assays were replicated using identical auxotrophic isolates to demonstrate reproducibility (Fig. S2), and for nine of the strains, two or three different auxotrophic mutants were isolated from the same parent strain to evaluate potential variability of transformation results due to allele-specific effects. For example, three independently

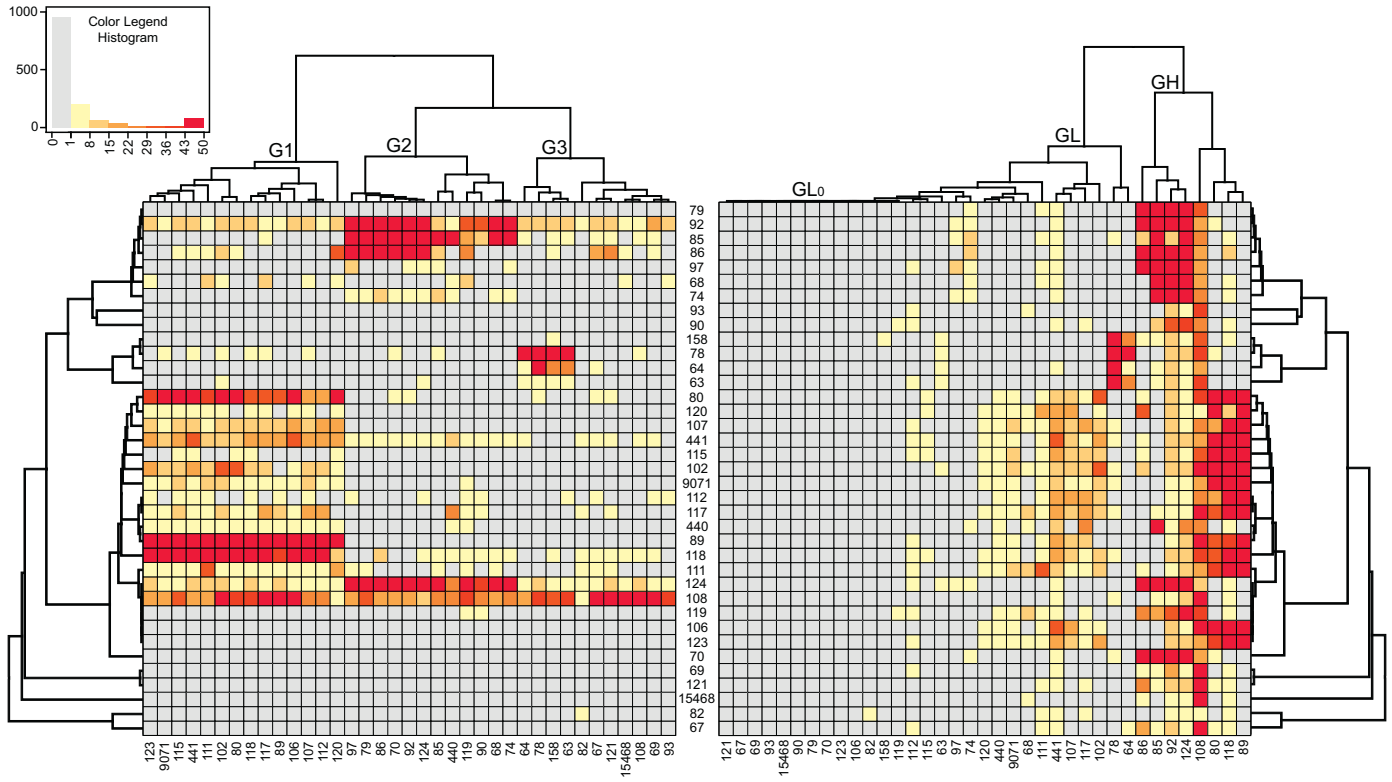

Fig. 5. Phylogenetic comparison of transformability and "donatability" among *Aeromonas* strains. Strains designated 9071 and 15468 are ATCC strains; all others are environmental isolates. Grids are heatmaps representing the numbers of transformant colonies yielded by transformation assays. Gray represents no transformation. Smallest nonzero values are light yellow; highest values are deep red. Rows represent strains arranged by phylogenetic relationships; trees superimposed over the vertical axes of the heatmaps are based on the ML phylogeny of *Aeromonas* (Fig. 6). For isolates with chimeric *gyrB* sequences, only the phylogenetic positions of the longest segments from the divided sequences are included for clarity. (a) Phylogeny vs. transformability heatmap depicts the abilities of auxotrophic recipient strains (rows) to be transformed by DNA from each wild-type (prototrophic) donor strain (columns) arranged by "donatability" group; dendrogram superimposed over the horizontal axis is based on the Ward's clustering of "donatability" (Fig. S6); the labels "G1," "G2," and "G3" designate "donatability" Groups 1, 2, and 3, respectively. (b) Phylogeny vs. "donatability" heatmap depicts the abilities of wild-type (prototrophic) donor strains (rows) to donate DNA to each auxotrophic recipient strain (columns) arranged by transformability group; dendrogram superimposed over the horizontal axis is based on the Ward's clustering of transformability (Fig. S5); the labels "GH" and "GL" designate high and low transformability groups, respectively; "GL0" designates nontransformers.

isolated mutants (a histidine auxotroph, a pyrimidine auxotroph, and a serine auxotroph) of strain 92 were tested (Fig. S3). These results (Figs. S2 and S3) demonstrated that there was no substantial variation between the different auxotrophic recipients derived from the same parent strain being transformed to prototrophy or between replicate assays performed with the same auxotrophic recipient. The assay of all auxotrophic strains indicated that not all aeromonad strains were capable of transformation; 10/37 (27%) could not be transformed under the test conditions established for this study (Figs. 5 and S4). Among the 27/37 (73%) strains that were competent for transformation, 9/27 yielded no more than 10 transformant colonies per plate; 5/27 had between 11 and 30 colonies on at least one plate, and 13/27 colonies had greater than 30 colonies on at least one plate (Fig. S4). DNA from all of the aeromonads could transform at least one other competent strain. *Aeromonas* strains 92, 108, and 124 were capable of taking up DNA from all 37 strains (Figs. 5 and S4).

The Ward's cluster analyses of the quick transformation assays for ability to (1) receive DNA and (2) donate DNA grouped isolates into transformation groups differently (Figs. 5, S5, and S6). The Ward's clustering based on ability to receive DNA (transformability) divided strains into two major groups, (1) a small group of high transformers (Group H) and (2) a large group including all nontransformers and low to moderate transformers (Group L) (Figs. 5 and S5). The transformability clustering solution yielded a cophenetic correlation coefficient of 0.95, which measures the degree to which the dendrogram of the cluster analysis preserves the original data (relative to 1). The Ward's clustering based on ability to donate DNA ("donatability") divided strains into three major groups of similar size defined primarily by the DNA preferences of the strongest transformers (Figs. 5 and S6). Roughly, these groups are (1) DNA preferred by isolates 80, 89, 102, 107 111, 117, 118, 120, 440, 441 and/or ATCC 9071 (Group 1); (2) DNA preferred by isolates 74, 85, 86, 92 and/or 124 (Group 2); and (3) DNA preferred by isolates 64 and/or 78 or not strongly preferred by any isolates (Group 3) (Figs. 5 and S6). This "donatability" clustering solution yielded a cophenetic correlation coefficient of 0.88.

Phylogenetic data analysis

Reconstructions of the relationships of the isolates in this study and their top BLAST hits from GenBank based on *gyrB* sequences recovered similar topologies using NJ with chimeras included and using MP and ML with chimeras split (Figs. 6 and S7). Both methods suggested that most isolates are members of two major clades. Clade I comprised *A. bestiarum*, *A. encheleia*, *A. media*, and *A. salmonicida* (Fig. 6). Clade II comprised *A. veronii* and *A. veronii* biovar sobria (or, in one case, its synonym *A. culicicola*) (Fig. 6). The analyses suggested that the remaining taxa, including *A. hydrophila*, *A. punctata*, and *A. schubertii*, form a basal grade (paraphyletic group) consisting of several smaller clades (Fig. 6). However, the cladistic MP and ML methods allowed the separate placements of partial sequences split from confirmed chimeras (Fig. 6). Further, despite the large amounts of missing data in partial sequences, bootstrap support

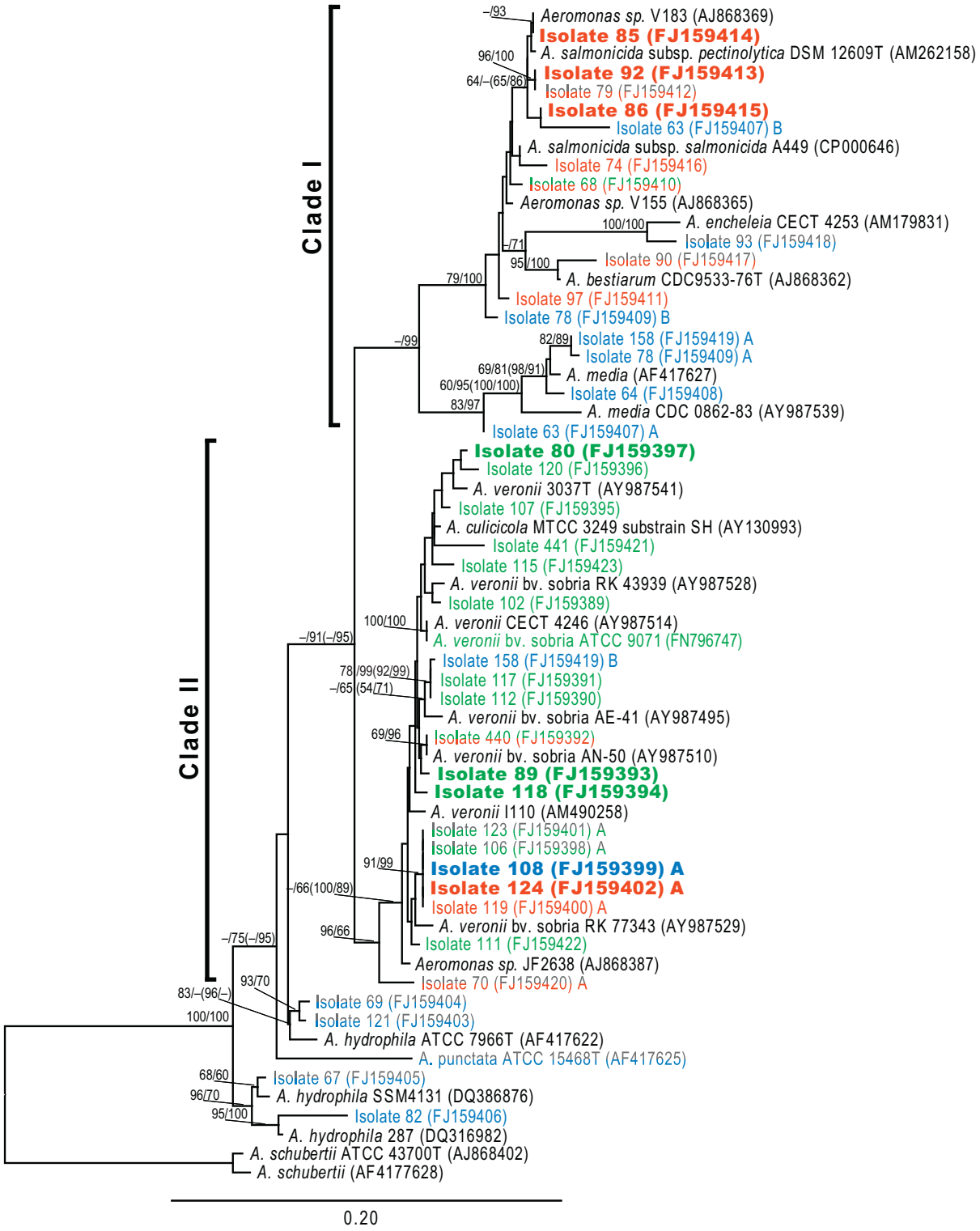

Fig. 6. Maximum likelihood (ML) cladogram from analysis of *gyrB* sequences from *Aeromonas* isolates used in this study and their best BLAST hits from GenBank. GenBank accession numbers are given in parentheses; a letter "A" or "B" following the parentheses indicates that the sequence is a segment derived from splitting a chimera. "A" indicates the longer segment; "B" indicates the shorter segment. Some "B" fragments were too short to include. Numbers above branches are maximum parsimony (MP) bootstrap proportions/maximum likelihood bootstrap proportions, respectively; numbers following in parentheses indicate increases in bootstraps ≥5% using the *gyrB* data set with partial sequence segments excluded. Only bootstraps for branches with at least one bootstrap support value ≥70% are shown. The scale bar represents nucleotide substitutions per site. High transformers (Group H) are listed in bold; low to moderate transformers (Group L) listed in regular font (Fig. S5). "Donatability" groups (Fig. S6) and preferred DNA sources of transformers (Table S1) are indicated by the following colors: Group 1 = green; Group 2 = red; Group 3 = blue; none = gray; when strains preferred to take up DNA from a group other than their own "donatability" group, taxon labels are two-toned, with preferred DNA color above and "donatability" group membership color below.

was substantially higher for clades in the MP and ML trees than in the NJ dendrogram (Figs. 6 and S7). The MP strict consensus topology (not shown) was consistent with the ML tree but had fewer resolved clades. Recombination analyses suggested that *gyrB* sequences from the following isolates were chimeric: 63, 70, 78, 106, 108, 119, 123, 124, and 158 (Fig. 6). Isolates 106, 108, 119, 123, and 124 all had an identical chimeric *gyrB* sequence. In the cases of isolates 63, 78, and especially 158, the phylogenetic placements of the split segments were both substantially different than the placement when included in analyses as a single chimera (Figs. 6 and S7).

Mantel tests of phylogenetic signal in strains' ability to donate and receive DNA represented by the Ward's cluster analyses gave different results. "Donatability" was significantly correlated with phylogenetic distance based on both ML and NJ measures of relatedness ($P < 0.0001$). In contrast, overall transformability was not significantly correlated with phylogenetic relatedness (vs. ML distances: $P = 0.831$; vs. NJ distances: $P = 0.7254$). Results were similar when all non-competent isolates were removed from the analyses (not shown). However, as stated above, the Ward's cluster analysis of transformability created groups of strains based primarily on levels of transformation, not type of DNA received during transformation. When only DNA preference was tested, the phylogenetic signal in strains' preferences was significant ($P < 0.0001$).

In agreement with the findings of the Mantel tests, the three "donatability" groups based on Ward's clustering (Figs. 5 and S6) are roughly consistent with groups found to have similar *gyrB* sequences (Figs. 5, 6, and S7). Thirteen of the 19 isolates assigned to Clade II plus ATCC 9071 belong to the same "donatability" Group 1 (Figs. 5, 6, and S6), and 5 of the remaining 6 isolates in Clade II from other "donatability" groups had chimeric *gyrB* sequences (Fig. 6). Similarly, 8 of the 15 isolates assigned to Clade I belong to "donatability" Group 2, and 5 of the remaining 7 from other "donatability" groups had chimeric *gyrB* sequences (Fig. 6). Four isolates (67, 69, 82, 121) and ATCC 15468 in the basal grade with *A. hydrophila* were assigned to "donatability" Group 3 (Fig. 6). However, many of the isolates with chimeric sequences in Clades I and II were also assigned to Group 3 (Fig. 6). In only one subclade did phylogenetic relatedness/sequence similarity poorly predict "donatability." Even though isolates 106, 108, 119, 123, and 124 have identical chimeric *gyrB* sequences, all three "donatability" groups occur among these five isolates (Figs. 5, 6, and S6).

Despite the nonsignificant Mantel test for phylogenetic control of overall transformability in *Aeromonas*, visual inspection of the transformability heatmap shows that some closely related and/or genetically similar isolates have similar patterns of DNA preferences (Figs. 5 and 6). For example, in Clade I isolates 85, 86, and 92 are close relatives that are generally able to transform DNA from "donatability" Group 2 (Figs. 5 and 6). Most of the isolates from Clade II are able to transform DNA predominantly from "donatability" Group 1, and the only exceptions to this pattern are isolates with chimeric *gyrB* sequences (Figs. 5 and 6). As with the phylogenetic patterns of "donatability," the clade of chimeric isolates 106, 108, 119, 123, and 124 exhibits the greatest contrasts among close relatives. Isolates 108 and 124 are two of the only three isolates (including isolate 92) in *Aeromonas* found to transform DNA from every potential donor. However, these two strains do not share the same donor preferences. Isolate 124 prefers "donatability" Group 2 and has an overall transformability pattern that is strikingly similar to that of isolate 92 from Clade I (Figs. 5 and S5). In contrast, isolate 108 prefers Group 1 and Group 3. Further, of the remaining three isolates with identical *gyrB* sequences, 106 and 123 were strict non-transformers, and 119 only weakly transforms DNA from itself and one other strain (Fig. 5).

Overall, the patterns of "donatability" group membership and DNA preferences of transformers are nearly identical among aeromonad strains (Figs. 5 and 6 and Table S1). The greatest difference between phylogenetic patterns of "donatability" and DNA preference is due to the complete lack of transformation by 10 of the 37 strains, preventing observation of DNA preference in these groups. However, among competent strains, only isolates 68 and 440 do not prefer to take up DNA from their own "donatability" groups. In contrast, strength of transformation shows no clear relationship to "donatability" groups and only a weak phylogenetic pattern. For example, high transformers (Group H) belong to all three "donatability" groups. However, the distribution of high transformers does not appear to be completely random with respect to phylogeny. Only one high transformer belongs to "donatability" Group 3, and half belong to Group 2. Further, 3 of the 4 high transformers in "donatability" Group 3 (isolates 85, 86, and 92) are close relatives in Clade I. None of the strains in the basal grade of the *gyrB* phylogeny were high transformers. Of these, all but isolate 82 failed to transform, and 82 could only take up its own DNA.

Discussion

Induction of competence

These data demonstrate that most members of the genus *Aeromonas* isolated from the environment are capable of natural transformation, but competence is induced under specific conditions. Not all aeromonads form transformant colonies under the conditions tested in this study. In vitro competence of transformable strains occurs when they are cultivated in dilute growth medium. This observation was also noted by Sakai [39] with *A. salmonicida* transformation and suggests that competence may be controlled by nutrient limitation. Induction of competence in *Aeromonas* occurs during late stationary phase and is in contrast to other transformable genera which become competent at different points during exponential phase or early stationary phase and maintain competence for a shorter period [2,8,32,53].

Aeromonas transformation can occur only in the presence of a threshold level of sodium and either magnesium or calcium. Other bacteria, such as *Mycobacterium* [31], *Azotobacter vinelandii* [32], and *Bacillus subtilis* [38], require magnesium or calcium for natural transformation to occur. The addition of magnesium to the transformation buffer does not indicate artificial transformation. Artificial transformation is usually achieved by osmotic or temperature shock in addition to calcium chloride treatment. This was not done in this study. Furthermore, the optimal temperature for *Aeromonas* transformation is around 30 °C, which coincides well with the optimal growth temperature for the mesophilic members of this genus. No special buffers or defined pH values are needed for the process to occur; high transformation frequencies can be obtained in distilled water that has been supplemented with sodium and magnesium indicating that if enough free DNA were present in aquatic habitats, aeromonad transformation could probably occur.

Phylogenetic methods

Phylogenetic reconstructions using *gyrB* sequences have provided insight into the nature of gene flow within and among the aeromonad taxonomic groups. However, the results of this study provide some basis for caution in interpretation of aeromonad phylogenies for both taxonomic purposes and for the development of hypotheses of trait evolution. Most significantly, taxa represented by chimeric DNA sequences will be incorrectly placed in phylogenies, especially when chimeras are combinations of distantly related gene lineages (e.g., isolate 158) [35]. Chimeric DNA sequences for both 16S ribosomal sequences and *gyrB* sequences

have previously been identified in aeromonads and are thought to have been acquired through horizontal gene transfer [42,45]. Although the ML method is expected to provide the most accurate reconstruction of phylogenetic relationships [12], this assumes that horizontal gene flow and recombination have not occurred. In this study, we found in preliminary analyses that ML actually performed worse than NJ when recombination was ignored, turning Clade II into a grade (paraphyletic group) (not shown). However, none of the methods correctly placed the *gyrB* sequence of isolate 158 when included as a chimera. This suggests that recombination and phylogenetic methods should be considered carefully in future taxonomic studies of *Aeromonas.* Because individual gene histories do not necessarily match the organismal history, future systematic studies of the evolution of natural transformation should benefit from use of multiple loci. Nevertheless, this single-gene study based on *gyrB* sequences has revealed substantial phylogenetic signal in the abilities of strains to donate and transform DNA.

Evolution of transformation

The patterns detected in this study suggest substantial genetic control over transformation and, in contrast with previous studies [39], provided strong evidence for phylogenetic signal in both "donatability" and transformability. However, nonsignificant Mantel tests comparing phylogeny to overall variation in transformability suggest that the relationship between phylogeny and transformability is more complex than between phylogeny and "donatability." This is likely due to differences in control mechanisms.

Mantel tests have confirmed that "donatability" is strongly correlated with phylogeny. We have defined "donatability" as the ability to donate DNA to another organism. In the process of transformation, DNA donation is expected to be passive since extracellular bacterial DNA is ubiquitous in the environment [25,33] and depends on the uptake abilities and preferences of the transformers (see reviews by [25] and [43]). In agreement with this assumption, all tested strains had the ability to donate, whereas only 27 of 37 could transform under the conditions provided. All strains in the study could donate because assays included a few tolerant transformers that could use DNA from all tested donors. Nonetheless, all of transformers had specific donor DNA preferences. Discrimination of donor DNA could occur through at least three mechanisms: (1) donor DNA could contain variable signal sequences that are recognized by uptake proteins in the transformers [44,52]; (2) restriction enzymes could destroy foreign DNA inappropriately methylated after uptake [10,58]; and/or (3) successful recombination between donor and recipient DNA molecules require sufficient sequence homology, which is unknown for *Aeromonas* species, but has been found to be as little as 20 base pairs in *E. coli* with recombination frequency dramatically increasing as length of homology increases, but decreases with mismatches within the homologous segment [56]. The simplest explanation for the strong correlation between "donatability" patterns and the *gyrB* phylogeny seems to be that "donatability" is controlled by overall genomic DNA sequence similarity between donor and transformer. This suggests that, despite the strong relationship between "donatability" and the *gyrB* phylogeny, it is possible that the relationship between "donatability" and the true organismal phylogeny is even stronger. Out of the 13 strains in Clades I and II that are not members of the same "donatability" groups as the majority of strains in their respective clades, 10 of the 13 had chimeric *gyrB* sequences (Fig. 6). The co-occurrence of *gyrB* recombination and unexpected "donatability" groupings suggests that the primary cause of inconsistencies between the *gyrB* phylogeny and "donatability" patterns is a mismatch between the *gyrB* sequence and the majority of the genome within these 13 strains. If this is true, then the "donatability" groups may actually estimate the organismal phylogeny better, at least at the deeper nodes, than the *gyrB* gene phylogeny does.

Superficially, Mantel tests suggest that transformability is not correlated with phylogeny, perhaps because transformability is not genetically controlled or because rampant gene flow has obliterated the phylogenetic signal even in the genes that control gene flow. However, contrasting results from a narrower test for phylogenetic signal in DNA preferences of transformers suggest some genetic control but that it is more complex than for "donatability." Because natural transformation is likely an active process, transformability could depend on the expression of multiple genes, causing complex patterns of evolution at the phenotypic level. As mentioned previously, variation in overall transformability among aeromonads appears to encompass at least two separate characteristics that could be controlled by different suites of genes. (1) Different related strains of aeromonads prefer to take up DNA from different "donatability" groups (Table S1). (2) Some strains are high transformers, and some take up little or no DNA under the study conditions. The phylogenetic signal tests and visual inspection of transformability patterns (Figs. 5 and 6) show that the DNA preferences of transformers are strongly controlled by phylogeny, much like overall "donatability." In fact DNA preference and overall "donatability" patterns are so similar (Fig. 6) that they likely have similar/shared control mechanisms, as described in the discussion of possible mechanisms for discrimination of donor DNA. One additional way that transformers could vary in preferences is in the level of stringency of discrimination. Such variation could be present in the function of uptake proteins, restriction enzymes, or mismatch repair systems, allowing transformation with less homologous donor DNA sequences to succeed more often in some recipients than in others [12,24,25]. However, visual inspection of transformability patterns (Fig. 5) shows that, when high transformers are compared to moderate transformers, the usage of preferred and nonpreferred DNA both generally increase or decrease by equal proportions, suggesting that strains differ most in gross uptake efficiency.

In contrast to DNA preference, our tests could not detect significant correlation between phylogeny and DNA uptake efficiency. For example, the nonsignificant Mantel test results were based on a transformability dendrogram (Figs. 5 and S5) that distinguished strains as high transformers and low transformers. Explanations for lack of phylogenetic signal in DNA uptake efficiency include: (1) loss of function mutations, (2) horizontal gene transfer, (3) lack of genetic control, and (4) low statistical power due to small sample size. Several strains in multiple clades failed to produce transformant colonies, suggesting loss of uptake function. However, when these strains were excluded, Mantel tests remained nonsignificant. Given the discovery of recombination in *gyrB* sequences, it is very likely that horizontal gene transfer has contributed to the erosion of phylogenetic signal in this study. Horizontal transfers have likely decreased the accuracy of the *gyrB* phylogeny as a proxy for the aeromonad organismal phylogeny, and genes controlling various aspects of transformability, including uptake efficiency, may also have been exchanged by distantly related strains. Because uptake efficiency may depend more on individual genes than overall genomic similarities, horizontal transfers of these genes could create greater impact on phylogenetic signal in uptake efficiency than in "donatability" and transformer preferences. For example, isolate 108 has high uptake efficiency similar to isolates 92 and 124 but DNA preferences unlike any other strains. It is possible that 108 has gained uptake genes similar to those of 92 and 108 through horizontal transfer. However, the previously confirmed horizontal transfer in the *gyrB* phylogeny has not been sufficient to hide phylogenetic signal in "donatability" and transformer preferences. Thus, it is unlikely that the rate of horizontal gene transfer has been sufficient to completely obscure phylogenetic signal in uptake

efficiency either. Alternatively, it is possible that uptake efficiency is not subject to substantial genetic control and that most differences between uptake efficiency among strains are environmentally controlled or random. However, all strains were subjected to the same environments during transformation assays, and reproducibility experiments suggest that uptake efficiency is not random. Further, visual inspection of transformability patterns also suggests that variation in uptake efficiency is not completely random with respect to phylogeny. Instead, we would suggest that the combination of horizontal gene transfer and low sample size for high transformers has resulted in a nonsignificant test for phylogenetic signal in transformability. For example, 3 or 4 of the 8 strains in Group H (isolates 85, 86, 92, and possibly 124) are close relatives (Fig. 6). Further, because full characterization of optimal transformation conditions for all aeromonads was beyond the scope of this study, transformation conditions were based on optimization with strain 92. Thus, it is likely that 3 of the 8 high transformers are closely related to isolate 92 because optimal transformation conditions are phylogenetically constrained. The low number of high transformers in this study may be due to substantial variation in environmental requirements for transformation among aeromonads. In agreement with the hypothesis that variation in uptake efficiency also contains phylogenetic signal, most of the non- or poorly-competent strains in this study are distant relatives of isolate 92 in the basal grade of *Aeromonas* (Fig. 6). This may also explain the unusual DNA preferences of the recombinant isolate 108. Isolate 108 prefers DNA from this basal grade (Fig. 5), suggesting that 108 may illustrate the DNA preferences that would be expressed by strains in the basal grade if they were provided transformation conditions that optimized their uptake efficiencies.

Gene flow in Aeromonas

Most evolutionists agree that reproductive isolation plays a key role in the formation and maintenance of biological diversity in nature [13]. Strong performance of cladistic phylogeny reconstruction methods and significant phylogenetic signal in transformation patterns suggest that aeromonads also tend to evolve as isolated, genetically independent lineages. Although natural transformation was found to allow gene exchange among all tested strains of *Aeromonas*, closely related strains are more likely to exchange DNA than distantly related strains. Related strains may also have similar optimal transformation conditions, strengthening the tendency for close relatives to acquire similar DNA during simultaneous transformation events. However, 16 (43%) of the aeromonad strains in this study were found to accept DNA from more than one "donatability" group, and this percentage could be an underestimate if most strains were tested under suboptimal conditions for transformation. Although recombination in *gyrB* has provided an obstacle to accurate reconstruction of the organismal phylogeny of *Aeromonas*, these recombinant sequences also reveal historical transformation events in natural environments. Recombinant *gyrB* sequences (from isolates 63, 79, and 158) show that horizontal transfer has occurred between distantly related strains in Clade I and between strains in Clades I and II (Fig. 6). Although unequivocal examples of historical gene exchange between close relatives were not discovered, these are not easily detected by analyses of recombination due to sequence similarities. Thus, our transformation results and *gyrB* sequence data are largely in agreement with the conclusion that horizontal gene flow can still occur between all of the strains but is genetically constrained.

Inheritance patterns of prokaryotes are often contrasted with eukaryotic inheritance due to (1) lack of sexual exchange within species and (2) occurrence of horizontal gene transfer between species [13]. However, the tendency for gene exchange among closely related aeromonads is reminiscent of the biological species concept that has been widely applied to eukaryotic evolution [13]. Although distant relatives are not completely barred from gene exchange in *Aeromonas*, molecular phylogenetic studies have also discovered surprising levels of horizontal gene transfer among eukaryotic species [11].

Conclusions

These data demonstrate that many different environmental isolates of *Aeromonas* are naturally transformable under the conditions tested. The optimal conditions for transformation correlate to those found in the natural environment. The finding that many aeromonads are naturally transformable provides an explanation for horizontal gene transfer among populations both in the past and present. However, most aeromonads prefer to accept DNA from close relatives. The patterns of transformation detected in this study suggest substantial genetic control over transformation that is phylogenetically constrained.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2013.01.004.

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