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Reservoir of Bacterial Exotoxin Genes in the Environment

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Abstract

This is the first report of an environmental reservoir of a bacterial exotoxin gene harbored in an atypical host in the environment. Screening bacterial isolates from the environment via *sea*-specific PCR identified an isolate with a DNA sequence >95% identical to the *Staphylococcus aureus* enterotoxin A gene (*sea*). 16S DNA sequence comparisons identified the environmental isolate as a Pseusodomonad. Laboratory studies confirmed that this Pseudomonad is psychrophilic. The results indicate that the *sea* gene is present in an alternative bacterial host, providing the first evidence for an environmental reservoir of exotoxin genes in bacteria. Transfer of these genes between phage and alternative bacterial hosts may promote the evolution of novel human diseases.

Exotoxins are secreted polypeptides involved in pathogenesis. Many exotoxin genes are carried on mobile elements such as phage. However, most epidemiological and ecological studies of infectious diseases have focused on the presence and activity of the bacteria per se, neglecting the potential role of the phage encoding the exotoxin genes. Phage are highly mobile genetic elements, have the potential to move between different environments, and are generally more resistant to environmental stress than their bacterial counterparts (*1-3*). Hence, phage can facilitate the transfer of exotoxin genes to non-toxigenic bacterial hosts, increasing the exotoxin gene pool. Moreover, transfer of exotoxin genes to new hosts may facilitate the evolution of novel human pathogens.

Sequence analysis of environmental metagenomes has shown that phage carrying exotoxin genes are common in the environment (the "free phage pool") *(4)*, but the normal bacterial hosts of the phage have not been identified in the same environments (*5*). A potential explanation of this finding is that phage in the environment can propagate in alternate hosts rather than those commonly associated with the human disease (Fig. 1).

To identify bacteria encoding the exotoxin genes, we screened bacterial isolates from environmental samples positive for the *Staphylococcus aureus sea* exotoxin sequence. Bacterial isolates were cultivated from the ambient environment by exposing Luria Bertani (LB) plates to the air and then incubating the plates at room temperature for 48-72 hours. The resulting colonies were sub-cultured into sterile 96-well plates and grown with aeration. Using *sea*-specific colony PCR, each of the isolates was screened for the presence of the *sea* gene. One putative *sea* positive isolate was single colony purified and the *sea* PCR was repeated to confirm the isolate was positive for the *sea* gene. The resulting PCR product was gel-purified, sequenced, and a BLASTN alignment against the non-redundant GenBank database was performed. The

alignment confirmed that the PCR product shared 95-96% nucleotide sequence identity with known *sea* genes. Most notably, the *sea* PCR product was 96% similar to a known *S. aureus* phage, ϕNM3. A multiple alignment and guide tree of the top BLASTN hits were produced using ClustalX2 (Fig. 2 and 3).

The environmental isolate did not grow on *Staphylococcus aureus* enrichment media at 35°C (*6*), but grew on LB at room temperature after 48-72 hr. Gram staining identified the isolate as a Gram negative rod. To identify the environmental isolate carrying the *sea* sequence, 16S rDNA colony PCR was performed and the PCR product sequenced. The resulting 16S sequence was imported into the ARB bacterial 16S rDNA database and its nearest relatives were identified (*7*). Phylogenetic analyses of the isolate, its nearest relatives, and select outgroups (including *Staphylococcus aureus*) were performed using the PAUP* program (Fig. 4) (*8*). These analyses provide robust statistical support for the inclusion of the *sea*-positive ambient air isolate in the genus *Pseudomonas*.

This report provides the first direct evidence that alternative microbial hosts can carry exotoxin genes. The evidence that there is an environmental reservoir of exotoxin genes in phage and in bacteria that are not normally associated with human disease suggests the possibility that novel diseases may evolve through horizontal transfer of virulence genes from phage to new microbial hosts (Fig. 1).

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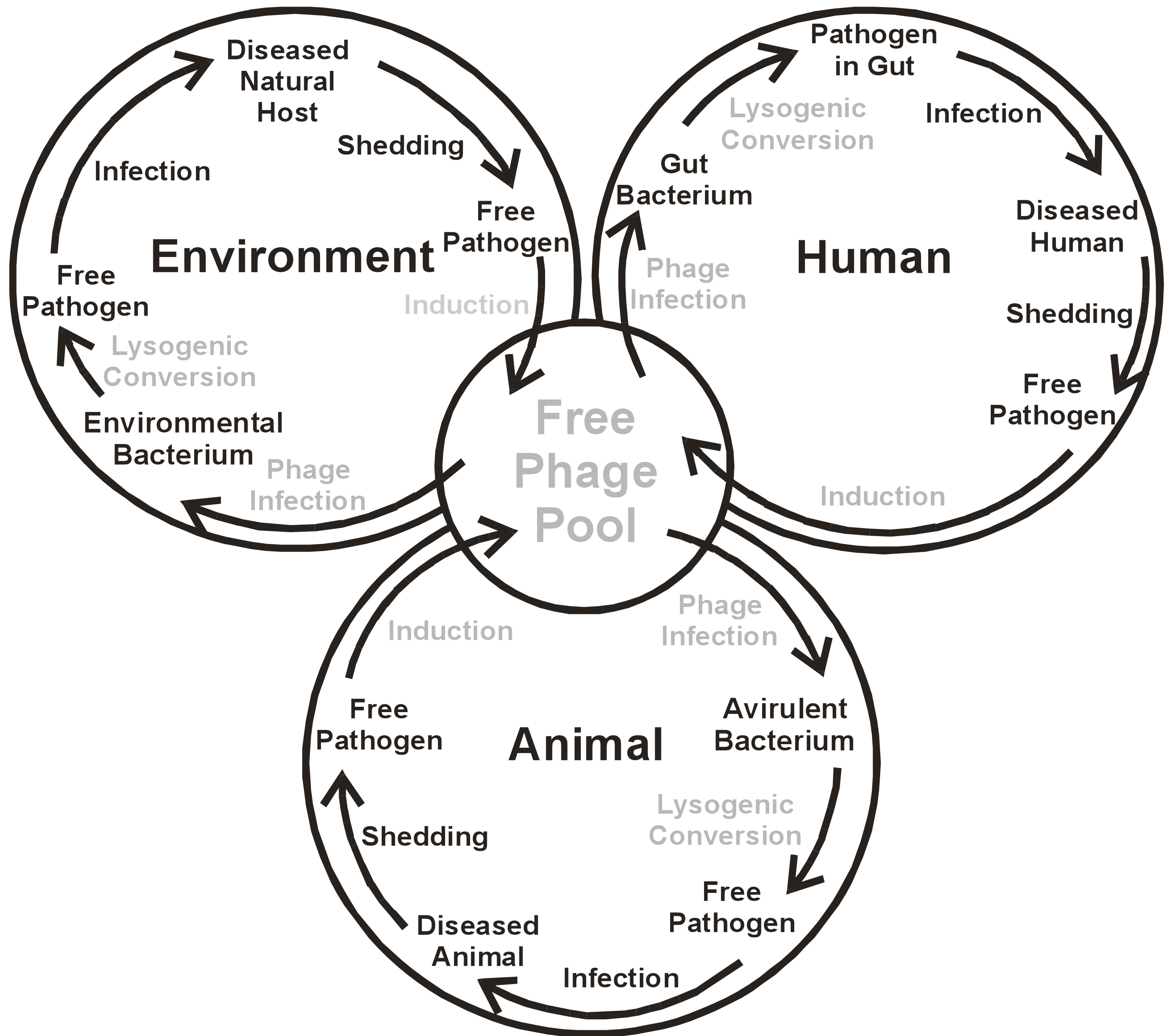

Fig. 1. Free phage pool of exotoxin genes. Proposed scenarios for how exotoxin-encoding phage might be maintained in the environment and produce human pathogens through genetic exchange between the free phage pool and the natural, human, and animal environments.

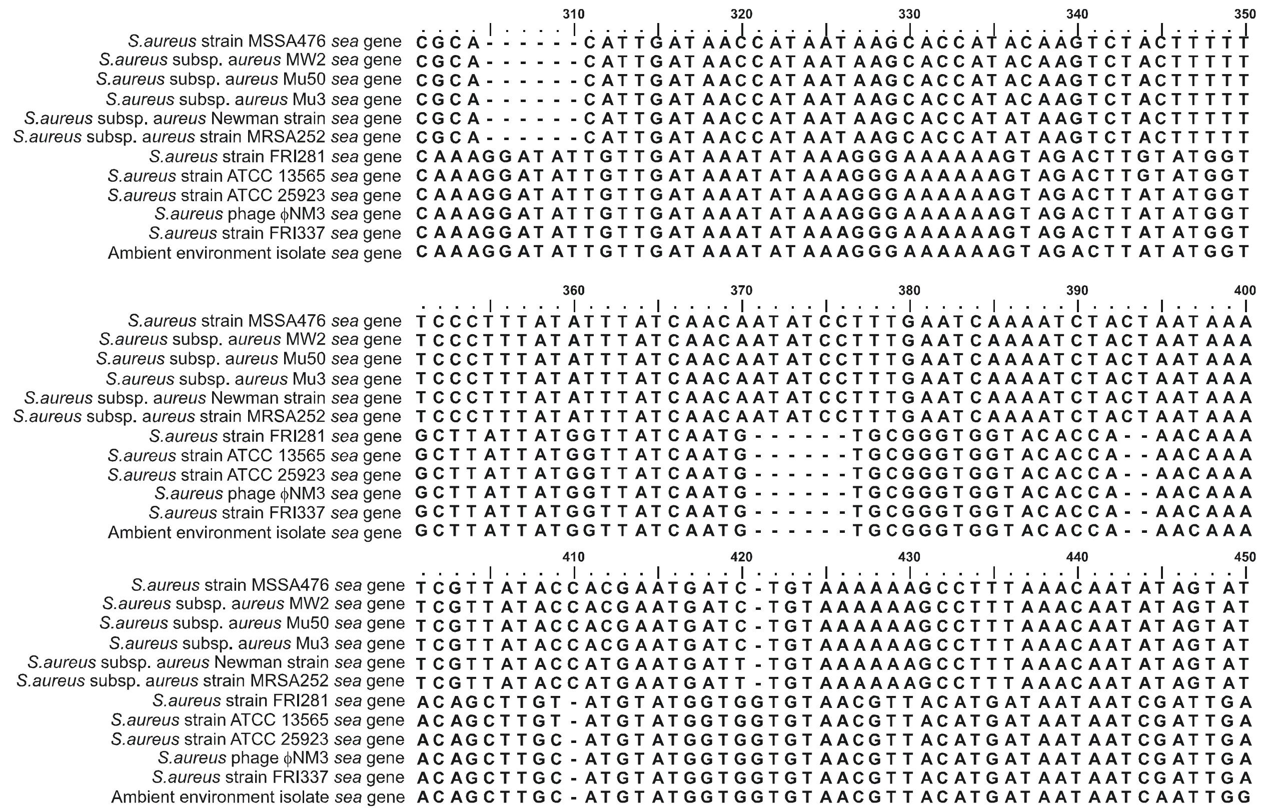

Fig. 2. ClustalX2 alignment of top BLASTN hits of the *sea* gene from the ambient environmental isolate. The *sea* PCR product amplified from the cultured ambient air isolate was verified against the GenBank non-redundant database. The FASTA files of the top hits were downloaded and aligned using ClustalX2.

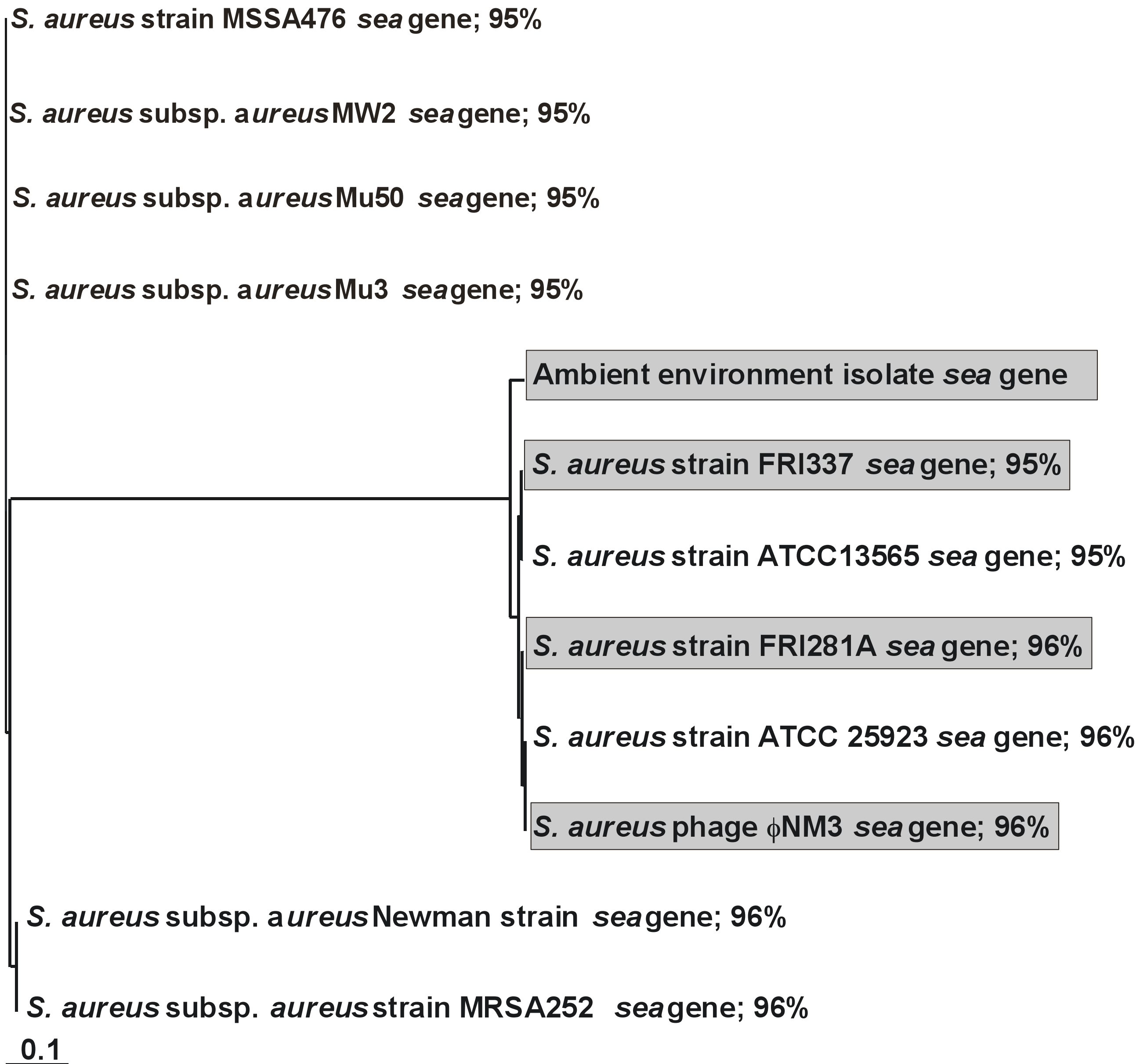

Fig. 3. ClustalX2 guide tree of the top BLASTN hits of the *sea* gene from the ambient environmental isolate. *S. aureus* strains FRI337 and FRI281A (Food Research Institute) are highlighted by the gray boxes because the positive control used for *sea* exotoxin PCR comes from *S. aureus* strain FRI913.

Fig. 4. Phylogenetic tree of *sea*-positive isolate, its nearest relatives, and select outgroups. We used Maximum Likelihood (ML) implemented in PAUP* to search for the highest likelihood tree under an HKY85 model of sequence evolution with estimated nucleotide frequencies, shape parameter and number of invariant sites. The heuristic search approach to find the best ML tree

included 100 random addition sequence searches using TBR branch swapping. The ML bootstrap involved 100 replicates with 10 random addition sequences searches per replicate. The best Maximum Parsimony (MP) tree was found through a random addition sequence heuristic search strategy with 100 replicates. The maximum number of trees kept during each search was capped at 1000. MP bootstrap analyses were performed using searches on 100 bootstrap replicated datasets using the same heuristic search strategy except with 10, rather than 100, search replicates. The Neighbor-joining (NJ) bootstrap analysis was performed with 1000 replicates. The *sea*-positive isolate is highlighted in bold and GenBank Accession numbers of each organism are in parentheses.