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Discrimination of *Campylobacter jejuni* Isolates by *fla* Gene Sequencing

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Comparison of the entire coding sequence of *flaA* (1,764 nucleotides) from 15 isolates of *Campylobacter jejuni* showed two regions of high variability, one region approximately from base positions 700 to 1,450 and a short variable region (SVR) from base positions 450 to 600. Parsimony analysis of the SVR sequences yielded a dendrogram similar to that which was derived by analysis of the entire gene. PCR was used to generate templates, and the SVR was sequenced with primers constructed to hybridize to conserved flanking sequences. The SVRs of 22 isolates of *C. jejuni* from four outbreaks that have been well characterized and a larger panel of isolates from three additional outbreaks were sequenced. Analysis of the nucleotide sequences produced results that grouped the isolates very similarly to other subtyping techniques. Sequence data were also generated for isolates from three additional outbreaks. Categorizing the isolates by *fla* SVR DNA sequence placed them in epidemiologically relevant groups. Sequence analysis of the *C. jejuni flaA* SVR may be a useful tool for epidemiologic investigations and could complement or replace serotyping and other subtyping methods.

Campylobacter jejuni is the most common bacterial cause of infectious enteritis in the United States (3). Infections are usually sporadic, but outbreaks associated with consumption of specific foods are common (13). Epidemiologic investigations of outbreaks often benefit from adequate subtyping techniques. Several methods have been used to subtype isolates of *C. jejuni*, 10 of which have been compared by Patton et al. (11). Those investigators concluded that multilocus enzyme analysis was the most discriminating method but that it was very labor intensive. Serotyping, either of heat-labile or heat-stable antigens (11), gave adequate levels of discrimination for epidemiologic purposes and is not technically difficult. However, the reagents required for serotyping are not readily available to most diagnostic laboratories.

The epitopes for the heat-labile serotypes are probably parts of proteins, and some, but not all, are part of the flagellin protein (14). The nucleotide sequences of the *flaA* gene encoding flagellin from four isolates of *Campylobacter* have been published (4, 6, 7, 9), and comparisons of those sequences have demonstrated variability within them. Variability within the *fla*-coding sequence has been used as the basis for PCR-restriction fragment length polymorphism (RFLP) methods for subtyping *C. jejuni* (8, 10). However, DNA sequence-based methods for subtyping have advantages over RFLP methods. More reliable results can be obtained in the face of interlaboratory variation, such as that due to gel mobility differences, and the relationships between different isolates can be more accurately deduced. This study was undertaken to explore the use of the DNA sequence of *fla* as the basis for subtyping *Campylobacter* isolates. A short variable region (SVR) was identified from the sequences of *flaA* from several isolates of *C. jejuni*, and the SVR was shown to be conserved among outbreak-related strains.

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MATERIALS AND METHODS

Bacterial isolates. DNA sequence data were derived for 67 isolates of *C. jejuni* used in this study (Table 1). Data for four isolates (three *C. jejuni* and one *Campylobacter coli* isolates) have been published previously (4, 6, 7, 9) and were also included in the analyses. The panel included 11 sporadic isolates not linked to described outbreaks; the remaining isolates were from seven investigated outbreaks. The first four outbreaks were associated with dairies, and isolates from these outbreaks were subtyped by 10 methods (11). Outbreak 5 was at a Wisconsin summer camp, and infection was associated with the consumption of tuna. Outbreak 6 was described by Birkhead et al. (2) and was associated with improperly pasteurized milk. Outbreak 7 was in a prison in New York State and was associated with the consumption of tuna fish salad. Four sporadic isolates were collected in surrounding counties at the time of outbreak 7. Isolates were serotyped for heat-stable and heat-labile antigens by standard methods (11).

Sequencing. The complete *fla*-coding sequences of isolates 81116 (9), IN1 (4), VC167 (7), and TGH9011 (6) were obtained from the GenBank database. The complete sequences of *flaA* were determined for 11 additional strains, as designated in Table 1. DNA templates from isolates whose entire *flaA* was sequenced were generated by PCR with the primers and protocols described by Nachamkin et al. (8). For most isolates, the PCR-generated fragments were inserted into the vector pCR II (Invitrogen, Inc., Carlsbad, Calif.), and three plasmids independently generated for each isolate were sequenced. The sequence from isolate A74/O was initially obtained from templates that were derived from clones in bacteriophage lambda *gt*11 and was repeated with PCR-generated template. PCR products from isolates SSU9894, SSU9896, and D1118 were sequenced without intermediate subcloning steps, since the complete sequences could be generated without the intermediate steps. Sequencing of the DNA templates was performed by dye terminator reactions (ABI-Perkin-Elmer, Foster City, Calif.) with the serial primers listed in Table 2. Not all primers produced usable results for all the isolates, but all segments of the amplification products were sequenced with at least threefold redundancy. Sequence data were gathered with an ABI 373a automated DNA sequencer (ABI-Perkin-Elmer). The sequence data were compiled with the AssemblyLIGN program (Intelligenetics Division, Oxford Molecular Group, Campbell, Calif.) so that the fragments were contiguous.

For isolates for which only a portion of *flaA* was sequenced for typing information, a template was also generated by PCR with the primers described above, and the PCR products were used directly for the sequencing reactions. The sequencing reactions for this group were performed with only a single pair of forward and reverse primers, FLA242FU and FLA625RU (Table 2), respectively, yielding a twofold redundancy.

Sequence data analyses. Sequences were aligned by the Pileup program in the GCG suite (5). The Plotsim program, also in the GCG suite (5), was used to generate similarity scores, which were converted to polymorphism scores by subtracting the similarity score from 1. Aligned sequences were compared and dendrograms were generated by using PAUP, version 3.1.1 (Phylogeny Analysis Using Parsimony) (12).

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences are listed in Table 1. Sequences that were identical to other sequences in GenBank were not submitted to the database.

TABLE 1. *Campylobacter* isolates, sorted in order of outbreak number following isolates not outbreak associated[a]

Isolate	Outbreak no.	Source	HL type	HS type	*fla* sequence no.	GenBank accession no.
81116[b]	NOA		6		20	J05635
A74/O[b]	NOA		5	36,23	19	AF015089
D2640[b]	NOA		50,5	23,36	21	AF015104
D2677[b]	NOA		5	36,23,15w	19	AF015105
D772[b]	NOA		9,53	5−,5+	10	AF015111
D935[b]	NOA		1,24	4,13	9	AF015112
IN1[b]	NOA		7		9	X57173
L17[b,c]	NOA		17		7	AF015113
L19[b,c]	NOA		19		12	AF015114
TGH9011[b]	NOA		36		11	Z29327
VC167[b,d]	NOA		8		14	M26945
SSU9896[b]	1	Cow (dairy A)	4	2	8	AF015115
SSU9894[b]	1	Cow (dairy A)	5	23,36,22	20	
EDL2	1	Cow (dairy A)	5	36,23	20	
EDL3	1	Cow (dairy B)	5	36,23	20	
EDL4	1	Cow (dairy B)	5	36,23	20	
EDL18	1	Human	5	36,23,22	20	
EDL22	1	Human	5	23,36,22	20	
SSU9892	1	Cow (dairy A)	5	23,36,22	20	
D224	2	Cow feces	4	2	3	AF015092
D226	2	Human	4	2	3	
D1118[b,e]	3	Cow milk	4	2	3	
D1108[e]	3	Cow feces	1	4	6	AF015090
D1114[e]	3	Cow milk	4	2	3	
D1117	3	Human	4	2	3	
D1159[d]	3	Pig, *C. coli*	44	46,15	4	AF015091
D445	4	Human	77	19	17	AF015106
D450	4	Human	77	19	17	
D452	4	Human	77	19	17	
D462	4	Bird	5	19,23,34	13	AF015107
D467	4	Bird	5	23,19,34	13	
D472	4	Bird	19,13	NT	16	AF015108
D473	4	Bird	NT	NT	15	AF015109
D5149	5	Human	ND	33	12	AF015110
D5150	5	Human	ND	33	12	
D5151	5	Human	ND	33,40w	12	
D5152	5	Human	ND	33,40w	12	
D5153	5	Human	ND	33	12	
D5154	5	Human	ND	33	12	
D2290[b]	6	Cow	4	2w	8	AF015102
D2286	6	Human	4	2	8	AF015100
D2287	6	Human	4	2w	8	
D2288	6	Human	4	2	8	
D2289	6	Cow	5	22,36,23	19	AF015101
D2291	6	Cow	NT	4,13w	18	AF015103
D2292	6	Human	5	36	19	
D2293	6	Human	4	2w	8	
D2248	7	Inmate	18	55	12	AF015093
D2249	7	Inmate	38	7,25,6w,18w	1	AF015094
D2250	7	Inmate	18	55(7)	12	
D2251	7	Inmate	18	55	12	
D2252	7	Food handler	18	55	12	
D2253	7	Sp	39	4,13w	2	AF015095
D2254	7	Inmate	18	55	12	
D2255	7	Inmate	18	55	12	
D2256	7	Inmate	18	55	12	
D2257	7	Inmate	18	55	12	
D2258	7	Inmate	18	55	12	
D2259	7	Inmate	18	55w	12	
D2260	7	Inmate	18	55	12	
D2261	7	Sp	4	2	8	AF015096
D2262	7	Sp	5	22,23w,36w	19	AF015097
D2263	7	Food handler	6,38	7,25,6w	1	AF015098
D2264	7	Food handler	18	55(7)	12	
D2265	7	Inmate	18	55	12	
D2266	7	Sp	10	8,17	5	AF015099
D2267	7	Inmate	18	55	12	

[a] NOA, not outbreak associated; HL, heat labile; HS, heat stable; w, weak reaction; NT, not typeable; ND, not done; Sp, sporadic isolate. All isolates from outbreak 7 were from a single outbreak among humans in three categories: inmates from a prison, foodhandlers in the same prison, and individuals from neighboring communities with sporadic cases at the same time.

[b] Strains used for total *flaA* sequence.

[c] Type strains for heat-labile serotypes.

[d] These isolate are *C. coli*; the rest are *C. jejuni.*

[e] Isolates D1118, D1108, and D1114 were all from the same cow.

TABLE 2. Primers used for PCR and dye terminator cycle sequencing

Primer[a]	Primer sequence (5′ to 3′)[b]	Purpose
FLA4F[c]	GGA TTT CGT ATT AAC ACA AAT GGT GC	Generate total gene template
FLA1728R[c]	CTG TAG TAA TCT TAA AAC ATT TTG	Generate total gene template
M13For	TGT AAA ACG ACG GCC CAG T	Sequence of cloned material
M13Rev	CAG GAA ACA GCT ATG ACC	Sequence of cloned material
FLA178F	ACT TTA GGT CAA GCT AT	Sequence portion of total gene
FLA178R	ATA GCT TGA CCT AAA GT	Sequence portion of total gene
FLA442F	CAA ATC GGC GCA AGT TC	Sequence portion of total gene
FLA442R	GAA CTT GCG CCG ATT TG	Sequence portion of total gene
FLA630F	GGA ACA GGA CTT GGA GC	Sequence portion of total gene
FLA630R	GCT CCA AGT CCT GTT CC	Sequence portion of total gene
FLA845AF	AAG ATA CCA CAG GTG TTG AAG C	Sequence portion of total gene
FLA845AR	GCT TCA ACA CCT GTG GTA TCT T	Sequence portion of total gene
FLA1133F	CCG ATG CTA TGG GAT TT	Sequence portion of total gene
FLA1133R	AAA TCC CAT AGC ATC GG	Sequence portion of total gene
FLA1290F	GGA TTT TCT GCA GGT TCA GG	Sequence portion of total gene
FLA1290R	CCT GAA CCT GCA GAA AAT CC	Sequence portion of total gene
FLA1463F	GCA GGT GTA ACT ACA CTT A	Sequence portion of total gene
FLA1532R	ATG TCG GCT CTG ATT TGA TC	Sequence portion of total gene
FLA1553R	TGA TTT TGT ACA GAA CC	Sequence portion of total gene
FLA242FU	CTA TGG ATG AGC AAT T(AT)A AAA T	Sequence the SVR
FLA625RU	CAA G(AT)C CTG TTC C(AT)A CTG AAG	Sequence the SVR

[a] The numbers in the FLA primers indicate relative positions on the *flaA* sequence; F indicates forward, and R indicates reverse.
[b] Spacing is for readability and not intended to show codon reading frames.
[c] Primers published by Nachamkin et al. (8).

RESULTS

Total *flaA* analyses. The *flaA* sequences of 11 isolates were determined and were aligned along with the four published sequences, giving a consensus sequence of 1,764 bases. Polymorphism scores for windows of 10 bases were determined along the length of the sequence and are illustrated in Fig. 1. An increasing polymorphism score is indicative of increased variability of that segment among the sequences analyzed. The *flaA* sequences were characterized by a relatively high level of variability between sequence positions 700 and 1450. A shorter segment of high variability (termed the SVR) was noted between positions 450 and 600. The SVR was flanked by regions of conserved sequence. Analyses for most parsimonious relationships were performed to compare the level of discrimination that could be discerned from the entire sequence of *flaA* versus the SVR. The results of this analysis are presented in Fig. 2.

SVR sequences. The SVRs plus flanking sequences totaling 267 bases from a collection of previously characterized isolates from four outbreaks were sequenced, aligned, and analyzed for the most parsimonious dendrogram (Fig. 3). The SVRs from isolates of *C. jejuni* from three other outbreaks were also sequenced. The sequences for all the isolates from outbreak 5 (Table 1) were identical. The serotypes and assigned SVR sequence types of the isolates from outbreaks 6 and 7 are also presented in Table 1.

DNA sequences representing all of the distinct types from all of the outbreaks were grouped into a single alignment and were analyzed for the most parsimonious relationships. The dendrogram that was generated (Fig. 4) had 21 terminal taxa. Each taxon was assigned a numerical designation, which represents a unique sequence, and the taxon number of the sequence for each isolate is indicated in Table 1.

All the isolates from outbreak 5 had identical SVR sequences, despite minor differences in heat-stable serotype. Analyses of the SVR sequences of the isolates from outbreak 6 segregated them into three groups. Isolate D2289, from a cow, and isolate D2292, from a human, differed slightly in their heat-stable serotypes, but they had identical SVR sequences. Isolate D2290, from another cow from the same farm, had the same serotypes and SVR sequence as all other isolates from humans. Isolate D2291 was also from a cow from the same farm from which isolate D2290 was derived, but its type was not represented among the isolates from humans. Sixteen isolates from outbreak 7 were analyzed along with four sporadic isolates collected from the same geographic region and time period. Two SVR sequences were identified among the outbreak strains. Outbreak isolates D2249 (7,25,6w,18w:38) and D2263 (7,25,6w:6,38) had similar heat-stable and heat-labile serotypes but identical SVR sequences. All of the remaining outbreak-associated strains had identical serotypes and SVR sequences. On the other hand, the sporadic isolates analyzed as part of outbreak 7 had four different SVR sequences and different serotypes as well.

DISCUSSION

We used comparative DNA sequencing to analyze a panel of outbreak-associated and sporadic isolates of *C. jejuni* in order to determine the utility of the procedure for the epidemiologic

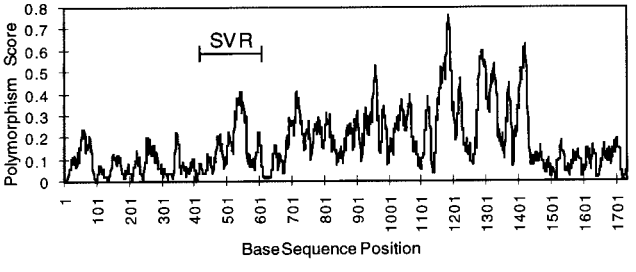

FIG. 1. Polymorphism scores for aligned *flaA* DNA sequences of 15 *Campylobacter* isolates. Similarity scores for a window of 10 bases were calculated by the Plotsim program of the GCG suite (5). The scores were converted to polymorphism scores by subtracting each similarity score from 1 and were plotted according to sequence position.

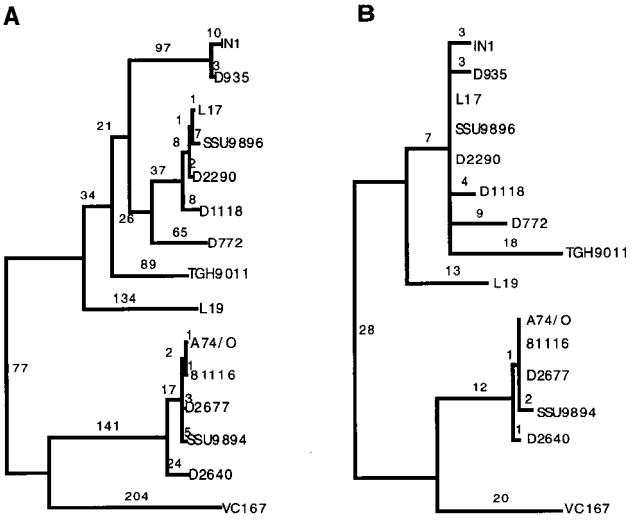

FIG. 2. Relationships derived from comparison of the entire *fla* sequence with the SVR sequence. Dendrograms of the entire aligned *flaA* sequences (A) and the aligned SVR (bases 450 through 600) of the *flaA* sequences (B) were generated by PAUP. Numbers along the horizontal lines are the numbers of nucleotide differences along the length of that line.

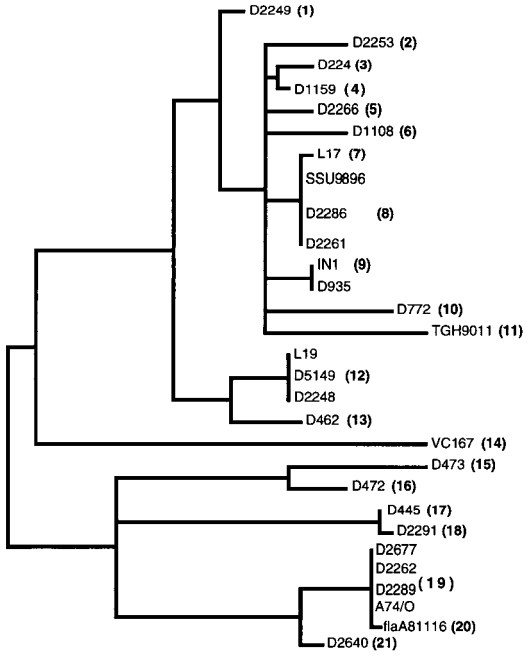

FIG. 4. Dendrogram of the aligned SVR plus flanking sequence totaling 267 bases of *flaA* from representatives of all sequences in all the groups of isolates tested. The dendrogram was generated by PAUP. *fla* sequence numbers were assigned on the basis of the position in this dendrogram and are indicated in parentheses to the right of the taxon designations.

characterization of strains. Multiple sequence analyses of the entire coding sequence of *flaA* from *C. jejuni* have demonstrated that there is as much as a 30% difference in the gene from one isolate to another. The variability is concentrated in two regions, a large variable region approximately from base positions 700 to 1450 and an SVR approximately from base positions 450 to 600 (Fig. 1). Analyses of aligned sequences to create dendrograms with the most parsimonious relationships showed that the degree of discrimination that can be detected with the SVR is similar to that which can be detected by using the entire *flaA* sequence (Fig. 2). Thus, the SVR segment was selected to test the utility of DNA sequence analyses for the characterization of *C. jejuni* isolates for epidemiologic studies. An advantage of analysis of the SVR is that it can easily be sequenced with a desired twofold redundancy over the entire region with a single pair of forward and reverse primers that bind to conserved flanking sequences, while analysis of the whole gene or a long variable region requires many more primers.

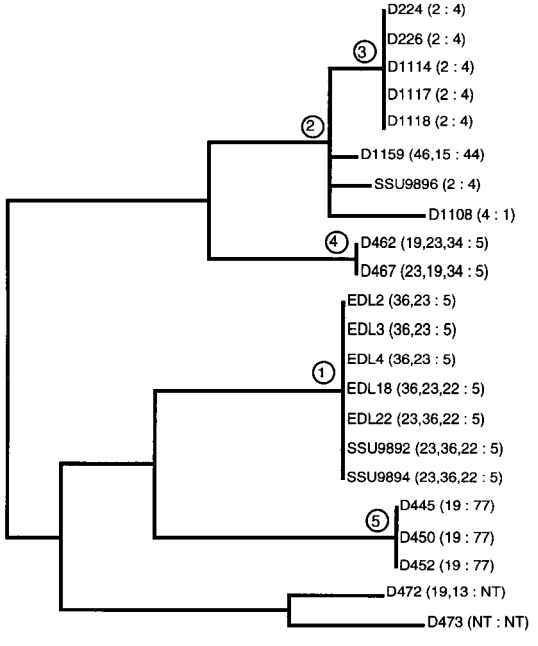

FIG. 3. Dendrogram of the aligned SVR plus flanking sequence totaling 267 bases of the *flaA* genes from a well-characterized pool of isolates of *Campylobacter* generated by PAUP. The circled numbers indicate clades (see Discussion). The taxa indicate the isolate designation followed in parentheses by heat-stable serotype : heat-labile serotype. Multiple serotype reactivity is indicated in order of the strength of the reaction, beginning with the strongest. NT, not typeable.

The results of SVR sequence-based typing of 22 well-characterized isolates are illustrated in Fig. 3. Patton et al. (11) have published the heat-stable serotype, heat-labile serotype, biotype, bacteriophage type, multilocus enzyme electrophoretic type, the results of restriction endonuclease DNA analyses and rRNA Southern blotting analysis, and the plasmid content for each of these isolates. In addition, these isolates have been characterized by PCR-RFLP of *flaA* (8). The greatest degree of discrimination in that study was achieved by analysis by multilocus enzyme electrophoresis (MEE) and restriction digest analysis of total genomic DNA. Sequence analysis of the SVR gave the same degree of discrimination. Dendrograms generated from the sequences of the SVR (Fig. 3) and MEE analysis (11) were similar. The members of clade 4 and clade 5, as labeled in Fig. 3, were from the same outbreak but had very different SVR sequences and were different by other subtyping methods (11). The members of clade 4 had the same heat-labile serotype and similar heat-stable serotypes as the members of clade 1 but differed by every other typing scheme as well as in their SVR sequences. This assortment of heat-labile serotypes independent of the flagellin sequence

indicates that at least some of the heat-labile serotypes are not a reflection of flagellin epitopes.

This study included two isolates of *C. coli*. The SVR sequences of these isolates did not segregate as outgroups. This is in contrast to data from the study by Aeschbacher and Piffaretti (1) in which dendrograms derived by typing by MEE indicated different groupings of the *C. coli* and *C. jejuni* isolates. Therefore, the genetic relatedness derived from the sequence of the SVR does not correlate with the genetic relatedness derived by typing by MEE. The data presented here are insufficient for making credible evolutionary conclusions, since the clonal quality of *Campylobacter* cannot be adequately evaluated. Data from studies with additional genes are required before evolutionary relationships can be accurate. However, it is noteworthy that all the members of SVR sequence types 8 and 19 had the same heat-labile serotypes and the same or similar heat-stable serotypes as the other members of the respective SVR type, even though they came from disparate sources. In both cases there were other isolates with different SVR types but the same serotypes. This may be indicative of the epidemic expansion of some clones.

The technology for generating DNA sequence data has become readily available for on-site laboratories or commercial service laboratories. With proper controls, sequencing can be expected to yield the same results in different laboratories even with the use of different methods. This, combined with the ease of generating a template for DNA sequencing by PCR, makes sequence-based subtyping a promising alternative to the more traditional methods. Serotyping requires very specialized reagents and does not always produce sufficient discrimination. Ribotyping and pulsed-field gel electrophoresis can be highly discriminatory but are labor-intensive. The PCR-RFLP assay is an alternative for typing of *Campylobacter* (8, 10). The apparatus for doing PCR-RFLP is extant in most laboratories where there is interest in subtyping bacteria, and the technique is easy. However, PCR-RFLP assays, as with ribotyping and pulsed-field gel electrophoresis, are difficult to standardize and interlaboratory comparisons of the results are especially difficult. Sequence analyses also give us more accurate information about the relationships among isolates.

Patton et al. (11) concluded that the heat-stable and heat-labile serotypes were useful for epidemiologic isolate discrimination. However, the reagents required for serotyping are difficult to make and control for quality. The genetic subtyping methods used by Patton et al. (11) were more discriminating than serotyping alone, but they were labor-intensive. Sequence-based typing with the SVR of the *flaA* gene has a discriminatory power similar to that of serotyping and, additionally, has the power to better determine relationships between nonidentical isolates.

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