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Application of *Streptococcus uberis* Multilocus Sequence Typing: Analysis of the Population Structure Detected among Environmental and Bovine Isolates from New Zealand and the United Kingdom

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We recently developed a multilocus sequence typing (MLST) scheme to differentiate *S. uberis* isolates and facilitate an understanding of the population biology of this pathogen. The scheme was initially used to study a collection of 160 bovine milk isolates from the United Kingdom and showed that the majority of isolates were from one clonal complex (designated the ST-5 complex). Here we describe the MLST analysis of a collection of New Zealand isolates. These were obtained from diverse sources, including bovine milk, other bovine anatomical sites, and environmental sources. The complete allelic profiles of 253 isolates were determined. The collection was highly diverse and included 131 different sequence types (STs). The New Zealand and United Kingdom populations were distinct, since none of the 131 STs were represented within the previously studied collection of 160 United Kingdom *S. uberis* isolates. However, seven of the STs were members of the ST-5 clonal complex, the major complex within the United Kingdom collection. Two new clonal complexes were identified: ST-143 and ST-86. All three major complexes were isolated from milk, other bovine sites, and the environment. Carriage of the *hasA* gene, which is necessary for capsule formation, correlated with clonal complex and isolation from clinical cases of mastitis.

Bovine mastitis continues to be the most economically important infectious disease of dairy cattle throughout the world (8, 11). *Streptococcus uberis* accounts for a significant proportion of subclinical and clinical intramammary infections in both lactating and nonlactating cows. An increasing prevalence of *S. uberis* mastitis has been reported, with approximately 14 to 26% of clinical cases in Canada, the United States, The Netherlands, and the United Kingdom being due to this pathogen (3, 9). *S. uberis* is also highly prevalent in Australia and New Zealand, where the dairy industry is mainly pasture based (13, 14). A recent report from New Zealand indicated that over 30% of all clinical mastitis was due to this organism (5).

Despite the high prevalence of *S. uberis* mastitis, its epidemiology is not completely understood. *S. uberis* has been isolated from many extramammary sites on the cow, including the skin surface, gut, tonsils, and genital tract (16). It is also found in high numbers in bedding material, which is a likely source of infection in housed cattle (2). It is assumed that teats become colonized by *S. uberis* present in the cow's environment and that this can lead to intramammary infection (for a review, see reference 12). Clarification of the importance of these environmental reservoirs to the incidence of mastitis is required for the development of better control measures for this pathogen. The ability to differentiate between specific strains of *S. uberis* is necessary for such epidemiological studies.

We recently described a multilocus sequence typing (MLST) scheme for this species, based on the comparative sequence analysis of internal fragments of seven housekeeping genes (4). An internet database was set up to provide worldwide accessibility. This MLST scheme was used to study the population biology of 160 bovine milk isolates from the United Kingdom. The strain collection contained 57 different sequence types (STs), showing that the population was heterogeneous. This is in agreement with a number of other studies using different typing methods to differentiate sets of *S. uberis* isolates (5, 10, 15, 17). However, the MLST analysis, unlike most previous typing methods, provided useful information about the genetic relationships between the strains. In particular, the majority of the isolates were shown to be related (112 of 160 strains were from the same clonal complex, designated the ST-5 clonal complex). Interestingly, all of the ST-5 complex isolates contained the capsule gene, *hasA*. The MLST analysis revealed that the population of *S. uberis* is not strictly clonal but is also evolving by recombination (4).

We describe here the MLST analysis of a collection of *S. uberis* strains isolated in New Zealand from a range of different sources. The aims of this study were twofold: first, to compare two populations from widely separated geographical locations, and second, to characterize isolates from different sources, including bovine milk, other sites on the cow, and environmental sites.

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TABLE 1. Allele frequencies of isolates from New Zealand and the United Kingdom

Allele	Allele frequency (%)[a]													
	arcC		*ddl*		*gki*		*recP*		*tdk*		*tpi*		*yqiL*	
	NZ	UK	NZ	UK	NZ	UK	NZ	UK	NZ	UK	NZ	UK	NZ	UK
1	23.72	49.38	64.82	70.00		2.50	6.32	55.63		6.25	66.01	78.13		8.76
2		24.38	14.62	9.38	38.34	69.38	60.08	30.00		68.13	3.56	8.75		39.38
3	9.88	6.88		9.38	14.23	9.38	13.83	7.50	4.35	6.25		6.25	67.98	25.63
4	13.83	5.63	4.74	6.25	11.07	8.13	14.62	5.00	1.19	5.00	22.92	6.25		0.62
5	0.39	4.38		0.62	21.74	4.38		0.62		4.38		0.62	0.39	1.25
6	11.86	1.88		1.25	0.79	3.13		0.62		1.88	4.74			5.00
7		0.62	1.98	1.25		1.88		0.62		2.50	0.39		1.58	2.50
8		0.62		1.25		0.62	1.58		1.58	0.62	1.19			1.25
9		1.25		0.62		0.62	0.39		3.16	1.25	0.79		0.39	1.25
10		0.62	4.74				1.19		1.98	0.62	0.39		4.74	12.50
11		0.62	0.39				1.19			0.62				1.25
12		0.62	3.56				0.39			0.62				0.62
13		0.62	0.39		9.88		0.39		10.28	1.25			4.74	
14		0.62	0.39		0.39					0.62			14.23	
15		0.62	0.39		0.39								3.16	
16		0.62	0.39		1.58				1.19				0.39	
17		0.62	1.58		0.79				57.71					
18	23.72		0.79		0.39				0.39				0.79	
19	8.70				0.39				0.39				0.39	
20	1.98		0.39						6.72				0.39	
21	2.37		0.39						2.37				0.39	
22	0.39		0.39						3.16				0.39	
23									1.19					
24	0.79								2.77					
25	0.39													
26	0.39													
27	0.39								0.39					
28	0.39								0.39					
29	0.39								0.39					
30									0.39					
31	0.39													

[a] The frequencies of the alleles of the seven MLST gene fragments in the collections of 253 New Zealand isolates (NZ) and 160 United Kingdom (UK) isolates are shown as percentages.

MATERIALS AND METHODS

Isolation and identification of *S. uberis* isolates. Putative *S. uberis* isolates were collected from different sources from five farms in New Zealand between 2002 and 2004. These were cultured on Trypticase agar supplemented with inulin and esculin, with bromocresol purple as a pH indicator. Esculin-positive colonies (observed by using a UV transilluminator) that fermented inulin (producing khaki-colored colonies) were provisionally identified as *S. uberis* and further identified by subculture onto Trypticase agar supplemented with mannitol and 4-methylumbelliferyl β-D-glucuronide with bromocresol purple as the pH indicator.

Isolates were confirmed as *S. uberis* by PCR amplification of the 16S rRNA gene with the primers 16S forward (5′-GAGAGTTTGATCCTGGCTCAGGA) and 16S reverse (5′-TTACCGCGGCTGCTGGCACGT), followed by sequence analysis (see below for PCR and sequencing methods). The sequence data obtained were compared to the sequence of *S. uberis* ATCC 27958 16S rRNA (accession number U41048) by using BlastN. Only isolates with 16S rRNA genes that were 100% identical to that of ATCC 27958 were included in the present study. Detailed information about the isolates, including their source, date of isolation, and, where appropriate, the number and disease status of the cow is available on the *S. uberis* isolates database at pubmlst.org/suberis. The isolates were entered into the database and given the following identification numbers: 239 to 308, 317 to 357, 362 to 447, and 449 to 504.

Chromosomal DNA purification, PCR, and sequence analysis. DNA purifications, PCR, and sequencing reactions were performed as described previously (4). Detailed protocols are also available at pubmlst.org/suberis/info/protocol.shtml.

Data analysis. Sequences were assembled from the resultant chromatograms as previously described (4). For each of the seven loci, each new sequence was assigned as a distinct allele. Each isolate is defined by an allelic profile, which corresponds to the allele numbers at the seven loci in the order *arcC*, *ddl*, *gki*, *recP*, *tdk*, *tpi*, and *yqiL*. Each unique allelic profile is assigned as an ST. An MLST database containing the sequence of all alleles, the allelic profiles (STs), and detailed information about the *S. uberis* isolates is maintained at Oxford University and can be found on the *S. uberis* pages of the MLST website (pubmlst.org/suberis).

The predicted evolutionary descent of the isolates was analyzed by using eBURST (eburst.mlst.net [6]). Statistical comparisons were performed by chi-square analysis of the raw data using Minitab 14 (table in worksheet option).

Detection of *hasA* gene by PCR. PCR amplification of the *hasA* gene (which is required for capsule formation) was performed as described earlier (4).

RESULTS

Allelic profiles of 253 New Zealand *S. uberis* isolates. The sequences of the seven gene fragments used for MLST were determined for the 253 isolates confirmed to be *S. uberis*. The sequences obtained were entered into the profiles database (pubmlst.org/suberis). Several novel alleles were identified for each of the seven genes compared to the 160 United Kingdom strains previously analyzed. A comparison of the allele frequencies of the New Zealand and United Kingdom isolates is shown in Table 1. There are some similarities between the allele frequencies, for example, *ddl* allele 1, *gki* allele 2, and *tpi* allele 1 were the most common alleles from both geographical locations. There were also some clear differences. For example, some alleles that were common in the New Zealand collection were absent from the United Kingdom collec-

TABLE 2. Characteristics of 253 New Zealand *S. uberis* isolates: distribution of ST, allelic profile, source, and carriage of *hasA*[a]

ST	No. of isolates	Source			Allele							*hasA* PCR
		Milk	Cow	Env.	*arcC*	*ddl*	*gki*	*recP*	*tdk*	*tpi*	*yqiL*	
ST-143 complex												
80	1	1			18	1	5	2	3	4	3	+
83	2	2			4	1	2	2	17	4	3	+
85	8	1	1	6	1	1	2	4	17	4	3	+
89	4	3	1		18	1	2	4	17	1	3	+
91	14	7	1	6	18	1	2	2	17	1	3	+
96	1	1			6	1	2	2	3	4	3	+
99	1	1			18	11	2	2	3	1	3	+
104	2	2			18	1	5	4	17	1	3	+
108	2			2	6	2	2	2	20	4	3	+
112	4			4	1	1	13	2	17	1	3	+
113	1			1	18	1	2	2	20	4	3	+
115	4		4		18	1	2	8	17	1	3	+
122	4		1	3	6	1	2	2	17	4	3	+
124	1			1	1	2	5	2	17	4	3	+
129	5	1	2	2	18	1	2	2	24	4	3	+
132	1	1			18	1	4	2	13	1	3	+
141	1	1			19	1	4	2	17	1	3	+
142	2	2			18	1	13	2	17	1	3	+
143	1	1			18	1	2	2	17	4	3	+
145	3	2		1	6	1	2	2	17	1	3	+
150	1			1	3	1	5	2	17	1	3	+
161	2			2	4	1	4	2	17	1	3	−
162	1			1	3	1	4	2	17	1	3	+
163	1			1	24	1	5	2	17	4	19	−
165	3			3	1	1	2	2	17	4	3	+
166	1			1	18	1	2	2	17	4	14	+
167	1			1	18	1	13	2	17	4	3	+
171	2			2	1	1	13	2	17	4	3	+
173	2			2	1	1	2	2	20	9	3	+
176	1			1	18	1	2	2	17	6	3	+
177	1			1	18	1	13	2	24	4	3	+
178	1			1	18	1	5	4	17	4	3	+
182	2		1	1	20	1	2	2	17	4	3	+
184	9		5	4	1	1	2	2	17	1	3	+
185	1			1	18	1	5	2	17	1	14	+
186	1			1	18	1	5	2	17	4	3	+
189	1		1		4	1	2	2	17	1	10	+
199	1			1	18	1	2	12	24	4	3	+
202	1			1	6	1	2	2	20	4	3	+
203	1			1	4	1	2	4	17	1	3	+
204	1			1	18	20	5	4	17	4	3	+
207	5	1	4		18	1	2	3	17	1	3	+
210	2		1	1	6	1	2	2	17	1	14	+
213	2		2		18	1	13	2	17	1	14	+
Subtotal	106	27	24	55								
ST-5 complex												
120	6		2	4	1	1	2	4	17	1	14	+
121	3		3		1	1	2	2	16	1	7	+
128	1	1			1	1	2	4	17	1	3	+
131	3	1	2		1	1	2	2	17	1	14	+
146	1	1			20	1	2	1	17	1	3	+
168	1		1		1	1	2	3	17	1	7	+
174	1			1	1	1	2	4	22	1	3	+
Subtotal	16	3	8	5								
ST-86 complex												
82	2	2			19	2	3	4	17	1	3	−
84	1	1			3	2	3	3	18	1	3	−
86	1	1			3	2	3	3	13	1	3	−
87	1	1			3	2	3	3	13	1	14	−
105	7	1	2	4	3	2	3	3	9	1	3	−
119	1			1	19	4	5	3	13	1	3	+
133	1	1			3	2	3	3	17	1	3	−
136	1	1			4	2	5	3	13	1	3	+

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TABLE 2—*Continued*

ST	No. of isolates	Source			Allele							*hasA* PCR
		Milk	Cow	Env.	*arcC*	*ddl*	*gki*	*recP*	*tdk*	*tpi*	*yqiL*	
140	1	1			3	2	4	2	17	1	3	+
147	1	1			3	2	3	3	13	2	5	+
148	1			1	3	2	3	2	3	1	3	−
152	1			1	3	2	3	4	13	1	3	−
160	1	1			19	2	3	3	9	1	3	−
164	1			1	6	2	3	2	13	1	3	−
172	1			1	19	2	5	3	13	4	3	−
179	1		1		3	4	3	3	13	2	3	−
180	1		1		4	2	19	3	4	1	3	+
181	1		1		6	2	3	1	13	4	3	−
193	1		1		3	2	3	3	17	4	14	−
197	1			1	20	10	4	3	13	1	3	+
Subtotal	27	11	6	10								
Others												
81	1	1			6	1	3	1	17	1	3	−
88	1	1			3	4	3	2	19	4	3	−
90	3	3			1	1	13	2	17	1	14	+
92	1	1			5	2	14	2	10	2	15	−
93	3	1	1	1	1	1	13	4	17	1	14	+
94	8	8			18	1	5	1	20	6	14	+
95	1	1			19	10	4	2	21	1	3	+
97	1	1			19	1	15	3	4	1	3	+
98	1	1			19	10	5	2	17	4	14	+
100	1	1			6	1	4	3	17	1	10	+
101	1	1			1	1	5	2	20	1	3	+
102	1	1			1	1	5	2	22	1	3	+
103	2	1		1	1	1	13	2	23	1	3	+
106	1	1			19	4	5	2	13	1	3	+
107	3	1		2	4	7	5	3	17	1	3	+
109	4		1	3	6	1	5	2	13	1	3	+
110	3		1	2	4	10	5	2	21	1	10	+
111	2		1	1	4	1	5	2	20	1	3	+
114	9	1	8		4	12	3	2	17	1	13	+
116	4		4		4	4	16	2	10	4	15	−
117	2		2		19	10	5	3	17	4	3	−
118	1			1	19	13	3	2	17	1	3	+
123	1			1	19	10	4	2	17	1	3	+
125	1		1		19	7	5	2	17	1	16	+
126	1	1			20	4	5	1	17	1	3	+
127	1	1			3	1	5	2	13	1	13	+
130	2	1	1		6	1	3	2	3	1	3	−
134	1	1			3	4	5	9	13	1	13	−
135	2	2			3	1	4	1	17	1	3	+
137	3	3			19	10	4	2	17	1	10	+
138	1	1			21	14	6	10	22	7	15	−
139	1	1			6	15	5	3	17	4	3	+
144	1	1			22	4	4	2	17	1	3	+
149	1			1	6	1	5	2	13	1	13	+
151	1			1	19	4	5	1	13	1	3	+
157	1			1	24	1	5	2	27	8	3	−
158	1			1	3	16	18	11	13	6	3	−
159	2	1		1	4	1	4	2	17	1	18	−
169	4			4	1	17	13	2	3	1	3	+
170	1			1	1	1	13	4	17	1	3	−
175	1	1			6	1	4	2	4	1	20	+
183	1		1		25	1	4	1	17	1	14	+
187	3		1	2	21	2	5	2	8	2	10	−
188	1		1		27	1	5	2	17	1	4	+
190	1		1		6	1	4	2	28	2	3	+
191	1			1	6	2	3	4	3	1	9	−
192	1			1	6	1	2	2	23	1	14	+
195	1			1	4	1	4	4	17	1	3	+
196	1			1	21	2	17	11	22	6	3	−
198	1			1	26	18	4	10	22	10	14	−
200	1			1	28	7	4	2	29	2	21	−

Continued on facing page

TABLE 2—*Continued*

ST	No. of isolates	Source			Allele							*hasA* PCR
		Milk	Cow	Env.	*arcC*	*ddl*	*gki*	*recP*	*tdk*	*tpi*	*yqiL*	
201	1			1	4	1	4	2	13	4	22	−
205	1	1			4	1	4	3	21	1	10	+
206	1		1		6	2	2	2	8	2	15	−
208	1		1		31	18	4	10	22	8	3	−
209	1		1		4	1	4	2	21	1	3	+
211	1		1		29	21	17	11	22	6	3	−
212	1		1		21	22	6	13	22	8	15	−
214	1		1		1	1	5	2	30	1	3	+
215	3	3			19	2	5	4	13	4	3	+
Subtotal	104	43	30	31								
Total	253	84	68	101								

[a] The STs are grouped into their clonal complexes. For each ST, the number of isolates and their source is shown (milk, other cow sites, or environmental). For further details of the sites of isolation, see the isolate database (pubmlst.org/suberis). The allelic profile of each ST is also shown, and the presence or absence of the *hasA* gene is indicated (+ or −). Env., environment.

tion, including *arcC* allele 18, *tdk* allele 17, *gki* allele 13, and *yqiL* allele 14. Other alleles, e.g., *arcC* allele 2, *tdk* allele 2, and *yqiL* alleles 1 and 2, were relatively common in the United Kingdom collection but absent from the New Zealand isolates.

In all, the New Zealand collection contained 131 different allelic profiles or STs. These were designated STs 80 to 152, 157 to 193, and 195 to 215. The allelic profiles and STs are shown in Table 2. None of these STs was present in the United Kingdom strain collection, indicating that the two populations are distinct.

The predicted evolutionary descent of the 253 New Zealand isolates was investigated with the clustering method, e-BURST, using the default group definition. This predicted that ST-143 was the founder of the largest group of related isolates (the bootstrap value for ST-143 being the primary founder was 80%). A second group of related isolates was identified centered on ST-86 (the bootstrap value for this primary founder was 77%). These two primary founders were entered into the database, which automatically assigns STs to clonal complexes (using the definition that all members of a clonal complex share four or more alleles with the primary founder of that complex). In total, three clonal complexes have now been identified: the ST-5, ST-143, and ST-86 complexes. The New Zealand isolates were assigned to the following clonal complexes: 106 isolates (representing 44 STs) were assigned to the ST-143 complex, 16 isolates (7 STs) were assigned to the ST-5 complex, and 27 isolates (20 STs) were assigned to the ST-86 complex. The other 104 isolates (60 STs) were not assigned to a clonal complex. The clonal complexes of the 131 New Zealand STs are indicated in Table 2.

Comparison of New Zealand and United Kingdom isolates. The clonal complexes of the 253 New Zealand isolates and the 160 United Kingdom isolates were compared. In the previous analysis of the United Kingdom isolates, only the ST-5 complex had been identified, but with the addition of two other primary founders to the database, some of the previously unassigned United Kingdom strains were assigned to the new clonal complexes (see the database at pubmlst.org/suberis). The percentage of all isolates from each country that were assigned to the different complexes is shown in Fig. 1. This graph also shows the percentage of New Zealand milk isolates in each complex. This was included to provide a direct comparison with the United Kingdom isolates, which were all isolated from milk. Clearly, the ST-5 complex is dominant in the United Kingdom, with the ST-143 complex being more predominant in New Zealand. However, none of the clonal complexes exclusively contained STs from one country.

A population snapshot of the 413 isolates from the United Kingdom and New Zealand, generated by using e-BURST, is illustrated in Fig. 2. In this diagram, single locus variants are joined, to illustrate the likely evolutionary descent of STs from their founders. There are several distinct clusters of STs, which are separated because they differ by at least two alleles. The three largest clusters are each centered on one of the three primary founders: ST-143, ST-5, and ST-86. It is worth noting that the clusters do not correspond exactly with the clonal complexes. This occurs for two reasons: some STs in a clonal complex do not have single locus variants and are therefore not depicted as part of a cluster, and some STs that are part of the cluster are not considered part of that clonal complex since they differ from the primary founder at more than three alleles. However, examination of the population snaphot showed that STs from one country generally cluster together (data not shown). For

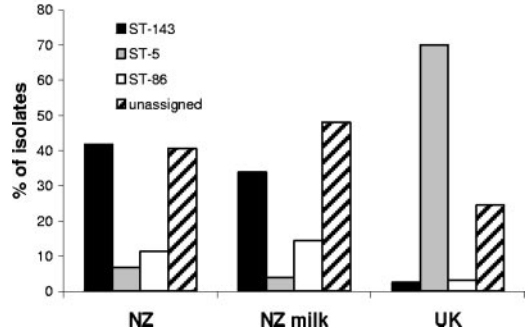

FIG. 1. Frequency of the clonal complexes in the United Kingdom and New Zealand. Results are shown for all New Zealand isolates, New Zealand milk isolates, and all United Kingdom isolates.

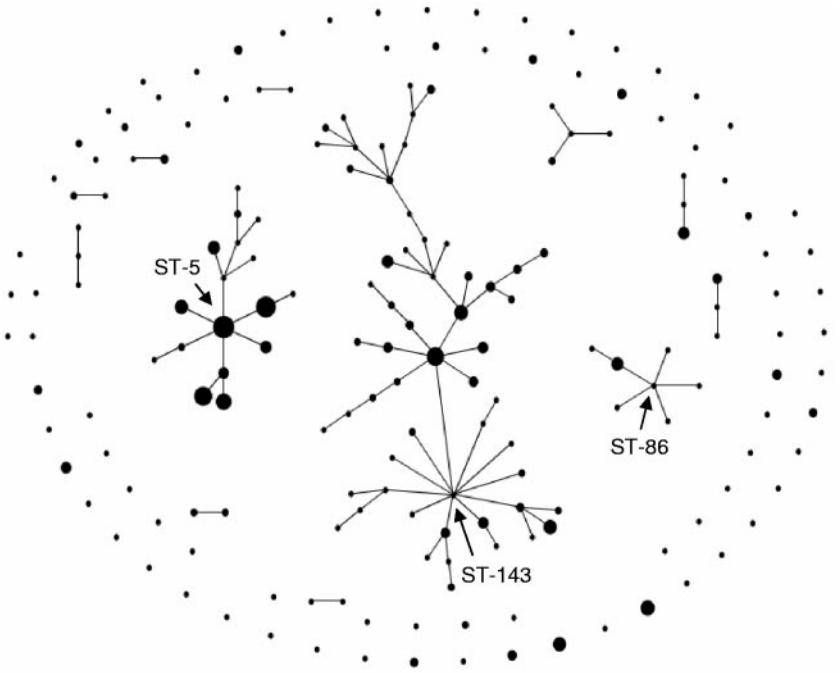

FIG. 2. e-BURST population snapshot of all 160 United Kingdom and 253 New Zealand isolates. The primary founders of the three clonal complexes are indicated. Other ST labels have been removed for improved clarity. The area of the circles correlates with the number of isolates with that ST. Single locus variants are joined by lines.

example, the largest cluster, containing ST-143, is almost entirely composed of STs from New Zealand, with the only exception being ST-12. Similarly, the cluster centered on ST-86 contains only New Zealand STs. In contrast, the second largest cluster, centered on ST-5, contains only United Kingdom STs.

Relationship between ST and source of isolation. The 253 New Zealand isolates were divided into three groups on the basis of their source. The isolates were from bovine milk (84 of the 253 isolates, or 33.2%); from other cow sites such as udder skin swabs, feet, or feces (68 isolates, or 26.9%); or from environmental sites such as race (a walkway for cattle) or paddock (101 or 39.9%). Further details of the isolation sites are available at pubmlst.org/suberis.

From the MLST results and isolate details, it was apparent that the strain collection contained a few duplicates. For example, several isolates were collected at the first milking when the infection was subclinical, and then the same ST was obtained from the same quarter a few days later when the infection had become clinical. Also, five isolates of the same ST were isolated from fecal samples from the same cow. To avoid sampling bias, these replicates were omitted from subsequent analyses (for the milk isolates, those from the first milking were omitted). The 11 isolates removed had the following identification numbers: 317, 321, 322, 329, 333, 491 to 494, 502, and 503. Thus, 242 isolates were included for the analyses shown in Tables 3 to 6.

The frequencies of isolation of each clonal complex from the three different sources are shown in Table 3. All three of the major complexes are clearly capable of establishing intramammary infections, as well as surviving in the environment and colonizing other bovine sites. Comparisons were made between the complexes of isolates in pairs of groups by chi-square tests. Statistical differences were observed between milk and environmental isolates ($P = 0.039$) and between cow site and environmental isolates ($P = 0.016$). In particular, the ST-143 complex was over-represented in the environment, and unassigned isolates were over-represented from milk and other cow sites. Isolates from milk and from other cow sites were not significantly different from each other ($P = 0.252$).

The frequencies of the most common STs (those isolated at least four times) were examined with respect to their source (Table 4). The most common ST within the collection was ST-91, which was isolated 13 times. ST-91 was common in both milk and environmental isolates and was also found once in the isolates from other cow sites. Other STs present in all three groups were ST-85, ST-105, and ST-129. However, a few STs were only present in one group. For example, STs 112 and 169 were only isolated from the environment, and STs 115 and 116 were isolated only from non-milk cow sites, specifically from fecal samples. ST-94 was unique to the milk group.

TABLE 3. Association between clonal complex and source of isolation

Source	No. of isolates of clonal complex (%)[a]				Total no.
	ST-143	ST-5	ST-86	Unassigned	
Milk	26 (33.8)	3 (3.9)	11 (14.3)	37 (48.0)	77
Cow sites	20 (31.2)	8 (12.5)	6 (9.4)	30 (46.9)	64
Environmental	55 (54.5)	5 (4.9)	10 (9.9)	31 (30.7)	101

[a] Milk versus cow site samples, $\chi^2 = 4.093$, $P = 0.252$; cow sites versus environmental, $\chi^2 = 10.261$, $P = 0.016$; milk versus environmental, $\chi^2 = 8.376$, $P = 0.039$.

TABLE 4. Association between ST and source of isolation[a]

ST	No. of isolates from:			Total
	Milk	Cow sites	Environment	
91	6	1	6	13
114	1	8	0	9
85	1	1	6	8
105	1	2	4	7
94	6	0	0	6
120	0	2	4	6
184	0	1	4	5
129	1	2	2	5
207	1	4	0	5
89	3	1	0	4
109	0	1	3	4
112	0	0	4	4
115	0	4	0	4
116	0	4	0	4
122	0	1	3	4
169	0	0	4	4

[a] The frequency of isolation and the source of the most common STs are listed. STs that were isolated at least four times are included.

Carriage of the *hasA* gene. The presence or absence of the hyaluronate synthase (*hasA*) gene, which is required for capsule production, was determined for each of the New Zealand isolates, and the results are indicated in Table 2. Carriage of *hasA* correlated with clonal complex (Table 5). The majority of *hasA*-negative isolates were in the ST-86 clonal complex or were not assigned to any complex. In contrast, all ST-5 complex isolates carried the gene.

Previous analysis of the 160 United Kingdom isolates (which were all milk isolates) had shown a correlation between *hasA* carriage and clinical mastitis (4), in line with a previous study of Danish isolates (7). For comparison, we analyzed the *hasA* carriage of the 77 New Zealand milk isolates relative to the disease status of the cows (Table 6). This analysis showed a similar trend, with 9.1% of isolates from cows with clinical mastitis and 27.3% of isolates from nonclinical cases being *hasA* negative. Furthermore, the majority of ST-86 clonal complex isolates from milk were from subclinical cases (8 of 11 [72.7%]), which is consistent with the finding that most isolates of this clonal complex are acapsulate.

DISCUSSION

Until recently, no suitable typing procedures were available to allow researchers around the world to carry out comparative population studies of *S. uberis*. We have developed a MLST scheme for this important veterinary pathogen that enables collections of *S. uberis* isolates to be directly compared at the genetic level (4). This was initially used to characterize a collection of 160 United Kingdom milk isolates. Here, we have extended this work by typing a large set of New Zealand isolates and compared this collection to the previously described collection of United Kingdom isolates. The New Zealand isolates were obtained from a variety of environmental sites, in addition to milk and extramammary bovine sites, providing useful information about possible sources of intramammary *S. uberis* infection.

TABLE 5. Association between *hasA* carriage and clonal complex of 253 New Zealand isolates

Clonal complex	No. of strains (%)		Total no. of strains
	hasA positive	*hasA* negative	
ST-143	98 (97.0)	3 (3.0)	101
ST-5	16 (100)	0 (0)	16
ST-86	6 (22.2)	21 (77.8)	27
Unassigned	68 (69.4)	30 (30.6)	98

TABLE 6. Clonal complex, disease status, and capsulation of 77 New Zealand milk isolates

Clonal complex	No. of strains					
	Clinical mastitis			Subclinical mastitis		
	hasA positive	*hasA* negative	Subtotal	*hasA* positive	*hasA* negative	Subtotal
ST-143	11	0	11	15	0	15
ST-5	0	0	0	3	0	3
ST-86	1	2	3	2	6	8
Unassigned	18	1	19	12	6	18
Total (%)	30 (90.9)	3 (9.1)	33	32 (72.7)	12 (27.3)	44

The 253 New Zealand isolates were shown to be highly diverse, with 131 different STs identified. This diversity was higher than that previously found in the United Kingdom milk isolates (57 STs in the collection of 160 isolates), which is likely to be due to the varied source of the isolates. Comparison of the STs identified within the two collections showed that none of the STs were common to both countries. This has probably resulted from a lack of movement of cattle between these nations. Interestingly, the allele frequencies of the two sets of isolates were markedly different, with some alleles being common in one country but completely absent in the other. These alleles have presumably evolved differently subsequent to geographical separation. This could have occurred by mutation or recombination, since our previous phylogenetic analyses of the seven loci showed evidence of recombination within the *S. uberis* population (4).

The majority of United Kingdom isolates had previously been assigned to one major clonal complex, called the ST-5 complex. A few of the New Zealand isolates (16 isolates and 7 STs) were also assigned to this clonal complex since they shared at least four alleles with ST-5. However, two new clonal complexes were identified in the present study: the ST-143 and ST-86 complexes. The ST-143 complex was the most predominant within the New Zealand population and contained 106 isolates and 44 STs, whereas the ST-86 complex contained 27 New Zealand isolates and 20 STs. Analysis of the previously unassigned United Kingdom isolates showed that a few of these shared at least 4 alleles with ST-143 or ST-86, and these were therefore assigned to one of these new complexes. Clustering analysis using e-BURST (Fig. 2) revealed that, although all three clonal complexes contained STs from both the United Kingdom and New Zealand, the closest genetic relationships were generally between STs from a single country.

Several of the most common New Zealand STs were isolated from the environment, as well as from extramammary bovine

sites and milk, which is consistent with intramammary infections resulting from contact with environmental reservoirs. In addition, all three complexes could be isolated from each of the three sources.

Carriage of the capsule gene, *hasA*, correlated with the clonal complex of the isolates, with all ST-5 and most (97.0%) ST-143 clonal complex members from the New Zealand collection carrying the gene. In contrast, a significant proportion of the ST-86 complex isolates (77.8%) and unassigned isolates (30.6%) were acapsulate. This *hasA* distribution was similar to that of the United Kingdom isolates, where all of the ST-5 complex isolates carried *hasA*, but some unassigned isolates lacked the gene (4). Thus, *hasA* appears to be enriched among the most prevalent clonal complexes in both the United Kingdom (the ST-5 complex) and New Zealand (the ST-143 complex).

Although acapsulate bacteria could be isolated from milk, demonstrating that they can establish intramammary infections, they were mainly associated with subclinical infections in both countries. Consistent with this, the ST-86 complex was disproportionately associated with subclinical mastitis. Interestingly, the carriage of *hasA* was also shown to correlate with isolation from clinical mastitis in a selection of isolates from Denmark (7). The significance of *hasA* gene carriage is unclear. The presence of capsule is not thought to play a direct role in pathogenicity, since mutation of *hasA* did not reduce pathogenicity in experimental infections (7). In addition, a few clinical isolates are *hasA*-negative. It therefore seems likely that carriage of *hasA* correlates with the presence of other genes involved in virulence. The high prevalence of the ST-5 and ST-143 complexes in the United Kingdom and New Zealand, respectively, suggests that carriage of *hasA* and/or other virulence genes may have contributed to bacterial survival and clonal expansion. A similar finding was reported for *Staphylococcus aureus*, in which the presence of both the *cna* and *tst* virulence genes was associated with prevalent lineages rather than sporadic clones (1).

In conclusion, we have shown by MLST that *S. uberis* has evolved into distinct populations in New Zealand and the United Kingdom. In total, three major clonal complexes have been identified, with highly different geographic prevalences. All three complexes were readily isolated from the environment, milk, and extramammary bovine sites. However, the observed differences in isolation rates of certain STs and complexes from clinical and subclinical cases of mastitis suggest that they may differ in their pathogenicity. Future work to determine which putative virulence genes in addition to *hasA* correlate with clonal complex and/or disease status will help to identify the bacterial factors associated with mastitis. Such studies should also facilitate the rational choice of suitable strains for vaccine development.

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