Curli Production and Genetic Relationships Among *Escherichia coli* from Cases of Bovine Mastitis

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ABSTRACT

Curli are adhesive surface structures produced by some *Escherichia coli* and *Salmonella* strains that bind host proteins and activate inflammatory mediators. In this study, 61 *E. coli* isolates from 36 clinical cases of bovine mastitis were characterized using enterobacterial repetitive intergenic consensus-PCR and screened for their ability to produce curli. Effect of curli production on case recovery, based on a return to precase milk yield, was investigated for a subset of 43 isolates from 20 quarters of 19 cows. Thirty-five (57%) of 61 isolates were curli positive. Fifty-eight of the 61 isolates clustered into 2 clonal groups at 52% genetic similarity. Genetically diverse *E. coli* isolates were simultaneously cultured from individual cases. Twenty-three isolates from 13 cows were clustered in clonal group I, of which 5 cases (38%) were curli positive; 35 isolates from 22 cows were clustered in clonal group II, of which 15 cases (68%) were curli positive. No association was found between genetic similarity and phenotypic curli expression of isolates from cows with clinical *E. coli* mastitis cases. Phenotypic curli expression in isolates did not affect recovery of cows' milk yield to premastitis production levels.

Key words: curli, *Escherichia coli*, fingerprinting, mastitis

INTRODUCTION

Curli are a unique group of thin, coiled, fibrous structures found on the surface of some bacteria. Expression of curli is regulated through both environmental and genetic interactions. Curli are capable of binding to fibronectin, laminin, and Congo red dye (Olsen et al., 1989) and play a role in the autoaggregation of bacteria and biofilm formation. In addition, curli enhance bacterial adhesion, invasion, and colonization and may con-

tribute to bacterial sepsis (Vidal et al., 1998; Bian et al., 2001; Gophna et al., 2001).

Curli were first described on *Escherichia coli* isolated from bovine mastitis cases (Olsen et al., 1989). Thin, aggregative fimbriae have been described in *Salmonella* spp. and are similar to curli in structure, function, and regulation (Collinson et al., 1991; Sjöbring et al., 1994; Römling et al., 1998). The genes required for curli formation in *E. coli* are encoded within 2 divergently transcribed operons: *csgBAC* and *csgDEFG* (Hammar et al., 1995). The curli structural subunit, known as curlin or CsgA, is secreted extracellularly and polymerized on the surface (Hammar et al., 1996). It has been shown that CsgD serves as an important transcriptional regulator of the *csgBAC* operon (Hammar et al., 1995; Römling et al., 1998).

In general, curli are produced in low temperature (<30°C) and low osmolarity conditions during stationary phase growth (Olsen et al., 1989, 1993; Maurer et al., 1998). When incubated at 26 to 28°C for 48 h in limited nutrient media containing Congo red dye, curli-producing colonies will usually display a red, dry, and rough morphotype, whereas curli-deficient strains often produce smooth, white, and moist colonies (Hammar et al., 1995, 1996). Altering environmental conditions such as NaCl content or temperature will cause some curli-producing bacteria to revert to the white phenotype (Olsen et al., 1993; Römling et al., 1998), although some strains are capable of expressing curli independent of temperature or other environmental factors (Römling et al., 1998; Bian et al., 2000; Uhlich et al., 2001). Curli expression in *E. coli* is variable among wild-type, laboratory, and pathogenic strains. Curli production was observed in 55% of bovine mastitis isolates (Olsen et al., 1989), 38% of non-O157 Shiga-toxin-producing *E. coli* (STEC) isolates, and is rare among O157 strains (Cookson et al., 2002).

Clinical studies in vitro and in vivo indicate that curli-producing bacteria are more pathogenic than curli-negative strains in their ability to adhere to, persist in, and invade host cells. Curli increased colonization and persistence of bacteria in avian colibacillosis, and similar strains demonstrated reduced persistence

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once their curli production was inhibited (la Ragione et al., 1999, 2000). Mice challenged with curli-producing *E. coli* variants displayed shorter survival times than those challenged with curli-deficient variants (Uhlich et al., 2002). Curli-producing *Salmonella* strains also achieved greater growth and yolk invasion in chicken eggs compared with their curli-deficient counterparts (Cogan et al., 2004). Curli facilitated internalization of *E. coli* by epithelial and HEp-2 cell lines, possibly mediated by fibronectin binding, where they may persist and evade detection by immune cells (Gophna et al., 2001, 2002). More recently, curli have been found to play a role in human sepsis (Nasr et al., 1996; Bian et al., 2000).

Curli may contribute to the pathogenesis of *E. coli* mastitis through several mechanisms. It has been shown that some bacterial strains can produce curli at increased temperatures, such as those encountered within the in vivo mammary environment. Curli may increase ability of bacteria to adhere to, colonize, or persist within the teat cistern and mammary tissue as well as evade the immune system. As proteins, curli may also activate immunologic pathways, increasing inflammation, recovery time, and clinical severity.

Targeting enterobacterial repetitive intergenic consensus (ERIC) sequences by PCR can be used to produce a unique genetic fingerprint for a bacterial agent. When compiling ERIC fingerprints into a dendrogram, groups or clusters of related isolates can be examined for common traits such as expression of a virulence factor and case outcome (Dopfer et al., 1999; de Moura et al., 2001). Should a particular bacterial group or strain be more likely to cause severe signs or possess a particular virulence trait, efforts to further explore, classify, and combat these strains can be focused to gain better insight into these organisms and their ability to cause disease.

In this study, *E. coli* isolated from clinical cases of bovine mastitis were characterized using ERIC-PCR and screened for their ability to produce curli by Congo red dye binding assay. Effect of curli production on case recovery, based on a return to precase milk yield, was also investigated in a subset of 19 cows.

MATERIALS AND METHODS

Origin and Confirmation of *E. coli* Isolates

Bacterial isolates were obtained from milk samples from clinical mastitis episodes in dairy cows enrolled in a clinical mastitis study. Cows were eligible for enrollment if they suffered from clinical mastitis as defined by having abnormal milk (discoloration, flakes, clots, or watery consistency) with or without presence of additional signs of inflammation (redness, swelling,

heat, and pain), plus any 2 of the following systemic conditions: abnormal temperature ($<37.8^{\circ}\text{C}$ or $>39.5^{\circ}\text{C}$), abnormal heart rate (>98 beats/min), abnormal behavior, sharply decreased milk yield (i.e., 20% or greater decrease in yield from previous day), dehydration (determined by skin tenting of eyelids), rumen contractions <2 per min, diarrhea, or recumbency. Diagnosis was made by the investigating veterinarian or his/her assistant. Cows with complicating injuries or other concurrent diseases were excluded from the study. Cows were treated according to study design and each farm's treatment protocols that included a combination of supportive care and antibiotics. Cows 528, 1167, 1450, 1525, 5403, and 12364 did not receive antibiotics.

Milk was collected on d 0 and 5 of the clinical mastitis event and shipped to a designated diagnostic laboratory for culture and identification of pathogens using National Mastitis Council methods (Hogan et al., 1999). Isolates identified as *E. coli* were sent to the Virginia-Maryland Regional College of Veterinary Medicine for inclusion in this study.

All isolates received were cultured on MacConkey agar plates (BD Biosciences, Franklin Lakes, NJ) and on Levine's eosin-methylene blue agar (Difco, Detroit, MI) for 12 h at 37°C . Isolates that produced dry, pink colonies on MacConkey agar and green, reflective colonies on eosin-methylene blue (i.e., positive reactions for lactose-fermenting coliforms) were classified as *E. coli*. Spot indole and oxidase tests (BBL, Cockeysville, MD) were performed on isolates with a weak or negative agar test result with indole-positive and oxidase-negative results identifying *E. coli* isolates. Isolates were cultured overnight in brain heart infusion broth (Remel, Lenexa, KS) combined with 30% glycerol (vol/vol), and maintained at -20°C for long-term storage. Stock cultures for daily use were maintained on modified trypticase soy agar II deeps (BBL) and maintained at 4°C .

Three diagnostic laboratories sent a total of 55 isolates from 29 cows. The Quality Milk Promotion Services Laboratory (Cornell University, Ithaca, NY) sent 36 *E. coli* isolates from 16 cows; the Colorado State University Veterinary Diagnostic Laboratory (Fort Collins, CO) sent 11 isolates from 6 cows; and the Kansas Veterinary Diagnostic Laboratory (Kansas State University, Manhattan, KS) sent 5 isolates from 5 cows. We determined that 3 isolates were not *E. coli* and excluded those from the study. An additional 9 isolates from 9 cows were received through the Production Management Medicine Program, Virginia-Maryland Regional College of Veterinary Medicine (Blacksburg, VA). In total, 61 isolates from 36 cows were included in this study.

Isolates were designated by site of origin (B, C, F, K, or V), cow number, and day cultured (d 0 or d 5). Thirty-

three of 36 Cornell isolates were cultured from 14 cows from one farm, designated 'B', with the remaining 3 isolates from 2 cows from other farms designated 'C'. Colorado isolates were designated 'F', and were all obtained from 1 farm. Kansas isolates were designated 'K' and Virginia isolates 'V'. Multiple isolates from the same cow were designated as a, b, c, or d. One cow (B1300) presented with 1 case of mastitis affecting 2 different quarters, yielding 2 isolates from each infected quarter. These isolates were differentiated by identifying left rear or right rear quarters. Duplicate isolates from the same quarter and sampling day were obtained from 17 quarter-sampling days in 13 cows.

Milk Yields and Mastitis Case Recovery

Daily milk yield was obtained for 19 cows (6 cows from site F and 13 cows from site B), milked 3 times daily from 5 d before (d -5) to 7 d (d 7) after the clinical mastitis episode. Forty-three of the 61 isolates examined were isolated from these 19 cows. Cows that produced $\geq 75\%$ of premastitis (d -5) daily milk yield by d 7 were considered to have recovered.

Curli Phenotype

All isolates were incubated at 26°C for 48 h (Hammar et al., 1995) on Congo red indicator (CRI) agar, which is YESCA agar supplemented with 20 mg/L of Congo red (Alfa Aesar, Ward Hill, MA) and 10 mg/L of Coomassie Brilliant Blue G dye (Tokyo Kaes Kogyo, Tokyo, Japan). Curli-producing *E. coli* bind Congo red dye and form red colonies on CRI, whereas curli-negative bacteria form white colonies. Stability of curli expression was determined by directly reculturing isolates onto fresh CRI and reincubating for another 48 h at 26°C.

DNA Extraction

Bacteria were grown in Luria-Bertani broth (BD Biosciences) at 37°C for 16 h in a Thermo Forma orbital shaker-incubator model 345 at 200 rpm (Thermo Forma, Marietta, OH). The DNA was extracted from 4 mL of culture using the Qiagen DNA Mini kit (Qiagen Inc., Valencia, CA) according to manufacturer's instruction.

csgA and *csgD* PCR

Genes for the curlin subunit protein, *csgA*, and the curli transcriptional regulator, *csgD*, were targeted and amplified in a PCR to determine their presence or absence in each isolate. Primers for *csgA* were selected as listed in Maurer et al. (1998). The *csgD* *csgDF* (5'-

ATGTTTAATGAAGTCCATAGT-ATT-3') and *csgDR* (5'-TTATCGCCTGAGGTTATCGTTTGC-3') primers were manually selected from the *csgD* listing in GenBank (accession number X90754; www.ncbi.nlm.nih.gov; NIH, Bethesda, MD).

Both the *csgA* and *csgD* PCR were performed in a total volume of 50 μ L with 300 ng of DNA, 20 pmol of each primer, and 45 μ L of Platinum PCR SuperMix High Fidelity (Invitrogen Life Technologies, Carlsbad, CA) in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf AG, Hamburg, Germany). The PCR amplification for *csgA* was as follows: denaturation at 94°C for 2 min, followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, and 30 s at 68°C with a final extension of 10 min at 72°C. The PCR reaction for *csgD* was cycled with the following protocol: denaturation at 94°C for 2 min, followed by 35 cycles of 30 s at 94°C, 30 s at 46°C, and 30 s at 68°C, with a final 10-min extension at 72°C.

The *csgA* and *csgD* PCR products were electrophoresed in 1% agarose gel buffered with sodium borate at 300 V for 10 min (Brody and Kern, 2004). Expected lengths of *csgA* and *csgD* PCR products were 200 and 650 bp, respectively. A randomly selected PCR product from each gene was sequenced at the Virginia Bioinformatics Institute at Virginia Tech (Blacksburg, VA) and confirmed as the target by comparing the result with each gene's GenBank entry.

ERIC-PCR

All strains were fingerprinted by ERIC-PCR using the ERIC2 primer sequence (Meacham et al., 2003). Reactions were carried out in a final volume of 25 μ L consisting of 100 ng of DNA, 25 pmol of ERIC2 primer, 2 U of Platinum *Taq* DNA polymerase (Invitrogen Life Technologies), 0.4 mM of each dNTP, and 5 mM MgCl₂ in a 1 $\times$ PCR buffer (provided with Platinum *Taq*). The ERIC-PCR reactions were conducted simultaneously on all isolates in a 96-well PCR plate in an Eppendorf Mastercycler Gradient thermocycler with the following protocol: denaturation for 2 min at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 57°C, 5 min at 72°C with a final extension step of 10 min at 72°C.

The ERIC-PCR products were electrophoresed in a 1.5% agarose gel buffered with Tris-borate-EDTA at 90 V for 2 h and 45 min. Gels were stained for 40 min with a 1 $\times$ solution of SYBR green (Molecular Probes, Inc., Eugene, OR) in a Tris-borate-EDTA buffer adjusted downward to pH 7.95, using 12 M HCl. Each gel contained 15 wells and was run with 3 lanes of 1 Kb Plus standard DNA ladder (Invitrogen Life Technologies; Figure 1).

The ERIC-PCR gels were illuminated with UV fluorescence and photographed with a Kodak Imaging Sta-

Figure 1. Enterobacterial repetitive intergenic consensus (ERIC) fingerprinting of 6 *Escherichia coli* strains isolated from clinical cases of bovine mastitis. M = DNA standard in bp; lane 1 = bacteria strain number 1167D0a; lane 2 = 1030D0b; lane 3 = 1080D5a; lane 4 = 1090D0a; lanes 5 and 6 = 1135D5a and 1135D5b, respectively.

tion 440CF (Eastman Kodak Co., New Haven, CT) for a 2-min exposure at f-2 through a #16, yellow, high-pass filter. Digital images were converted and exported to TIFF format using Kodak 1D Image Analysis Software v. 3.5.5.B (Eastman Kodak Co.).

Statistical Analyses

The TIFF images of ERIC-PCR results were standardized and analyzed using GelCompar II software (Applied Maths, Inc., Austin, TX). Similarity matrices were constructed using Pearson correlation coefficients based on densitometric readings of the banding patterns. Isolates were clustered based on their similarity coefficients using unweighted pair group method analysis. One dendrogram was constructed using data from all isolates. A second dendrogram was constructed by the same method using data from the 43 isolates from 19 cows for which milk yield records were available.

Association of cluster groups with presence or absence of curli production in cows, and with return to precase milk yields, as well as association of use of antibiotics with return to precase milk yields, were determined by Fisher's Exact tests using Simple Interactive Statistical Analysis (SISA) online statistical

analysis software (Uitenbroek, 1997; <http://home.clara.net/sisa>). Exact *P*-values are presented.

RESULTS

Phenotypic Curli Expression

Sixty-one *E. coli* isolates were cultured on CRI agar. Thirty-five isolates (57%) expressed the red phenotype (curli positive) and 26 (43%) expressed the white phenotype (curli negative). Neither variant (red or white) altered its phenotype when recultured on CRI agar.

When more than 1 isolate was obtained from a cow, those isolates displayed the same curli phenotype, with 1 exception. Two isolates cultured on d 0 from cow B303 (B303D0a, B303D0b) were curli negative, whereas a third isolate obtained on d 5 (B303D5c) was curli positive. Curli-positive *E. coli* isolates were cultured from 21 cows, and curli-negative strains were cultured from 15 cows.

Presence of *csgA* and *csgD* Detected by PCR

The curlin subunit gene, *csgA*, and the curli transcriptional regulator gene, *csgD*, were both present in all isolates, regardless of phenotype expressed.

ERIC-PCR Dendrogram

At a threshold of 52% similarity, 58 *E. coli* isolates were clustered into 2 clonal groups. Group I contained 23 isolates from 13 cows, and group II contained 35 isolates from 22 cows. Three isolates were genetically distinct from the isolates in the 2 clonal groups: V3758D0a shared 49.9% genetic similarity with isolates in the 2 groups, and isolates B1290D5a and B1290D5b had only a 6.7% similarity with the 2 groups, but were 87.7% similar to each other (Figure 2).

A single *E. coli* isolate was obtained from 22 mastitis cases, whereas multiple *E. coli* isolates were obtained from 14 mastitis cases such that 2 "sibling" isolates were obtained from 7 cases, 3 isolates from 3 cases, and 4 isolates from 4 cases. In 7 of those 14 cases, sibling isolates possessed $\geq 90\%$ similarity to each other and were clustered closely. In the remaining 7 cases, at least one of the sibling isolates was $< 90\%$ similar and clustered on a different or slightly more distant branch. In total, 28 of the 39 isolates clustered at $\geq 90\%$ similarity to isolates within the same cow. Isolate B1167D0a was 85% similar to isolates B1167D5b and B1167D5c, which were 95% similar to each other. Isolate C1030D0a was 86% similar to C1030D5b, but also was more closely related to, and clustered with, B1090D0a and B1080D5a. Isolates F1300LRD0c and F1300LRD0d were only 83% similar to each other and

Figure 2. Dendrogram of 61 *Escherichia coli* isolates from 36 cows with clinical mastitis. Two distinct groups form at 52% genetic similarity. Curli phenotype is indicated by + or -.

placed in separate subclusters, and, in addition, were only 66% similar to isolates F1300RRD0a and F1300RRD0b. Isolates F12544D0a and F12544D0b were 81% similar and, as a result, clustered on different branches. Isolates B303D0a and B303D0b, 2 curli-negative isolates, were placed in group I and were 99% similar, whereas B303D5c, a curli-positive isolate, was placed in group II with an average similarity of 52% to group I. Isolate B1450D0a was only 60% similar to its sibling isolates B1450D0b, B1450D5c, and B1450D5d, which shared greater than 96% genetic similarity with each other.

A separate cluster analysis was performed and a dendrogram produced for the 43 isolates from 19 cows for which milk yield records were available. This dendrogram also divided isolates at a threshold of 52% similarity into 2 distinct groups (III and IV). Group III consisted of 18 isolates from 8 cows, and group IV of 23 isolates from 11 cows. Isolates B1290D5a and B1290D5b were located outside of these 2 groups and possessed only 6.7% genetic similarity to these groups (Figure 3).

Curli Distribution by ERIC-PCR

Curli-positive *E. coli* isolates were present in both groups in both dendrograms (Figures 2 and 3). Five of 13 cows (38.5%) in group I and 15 of 22 cows (68.2%) in group II ($P = 0.157$) had 8 and 26 curli-positive isolates, respectively, with 15 and 9 curli-negative isolates in groups I and II, respectively (Figure 2 and Table 1).

Mastitis Case Recovery

Eight of 19 cows (42%) with milk yield records were considered to have recovered, regaining $\geq 75\%$ of pre-mastitis (d -5) daily milk yield by d 7. Of the remaining 11 cows, 8 cows regained 36 to 69% of their pre-mastitis daily milk yield by d 7, 2 cows died within 24 h of detection of severe clinical signs, and 1 cow died within 7 d of onset of severe clinical mastitis (Table 2). Recovery was not affected by antibiotic treatment because 5 of 8 cows (62.5%) with $\geq 75\%$ return to pre-mastitis milk yield, and 8 of 11 cows (72.7%) with $< 75\%$ return to pre-mastitis milk yield received antibiotics.

Three of 8 cows (37.5%) in group III and 4 of 11 cows (36.4%) in group IV were categorized as recovered (Figure 3; Table 2). Three of the 8 cows (37.5%) in the recovered group and 8 of 11 cows in the nonrecovered group (72.7%) were classified as curli positive (Tables 1 and 2), with cow B303, which yielded curli-negative isolates on d 0 and 1 positive isolate on d 5, counted as a curli-positive case.

Figure 3. Dendrogram of 43 *Escherichia coli* isolates from 19 cases of mastitis from cows with production records. Two distinct groups form at 52% genetic similarity. Curli phenotype is indicated by + or –.

DISCUSSION

Overall incidence of curli expression in all 61 isolates was 57%. This is similar to the 55% incidence initially reported in the first report of curli fibers and the only published report of curli expression in *E. coli* from cases of bovine mastitis (Olsen et al., 1989). In addition, Olsen et al. (1989) reported curli expression in 55% of bovine fecal isolates. None of the curli-negative or curli-positive isolates changed its phenotype after successive culturing on CRI agar.

Rate of curli expression in *E. coli* has been shown to be related to bacterial behavior, host, and environment, and varies from rare to 100% (Cookson et al., 2002; Goldwater and Bettelheim, 2002). Similar rates of expression between fecal and mastitic isolates as shown in our study and the study by Olsen et al. (1989) support the idea that mastitic *E. coli* are opportunistic, environmental bacteria. These data also indicate that curli expression may be a stable phenotype among the mastitic *E. coli* investigated, because repeated culture of white (negative) variants did not cause them to express the red (positive) phenotype, and vice versa. It should be noted, however, that curli expression was observed under ideal growth conditions, and such stability may not be the case in the changing, in vivo environment of the udder.

All isolates possessed the genes for the curlin subunit protein, *csgA*, and the curli transcriptional regulator, *csgD*, regardless of phenotype expressed. Similar findings have been reported (Maurer et al., 1998; Zogaj et al., 2003), and our findings support the claim that both genes are ubiquitous in all *E. coli* strains. In this experiment, only presence of the *csgA* and *csgD* gene was evaluated, and it is possible that, although present, either gene was not expressed or activated in white variants. Another gene required for curli expression also may be missing, altered by insertions or deletions, or not be expressed. The ubiquitous nature of the *csg* genes does not allow these genes to be used in screening for curli-producing *E. coli*. At this time, it seems that physical evaluation of the binding of Congo red dye (or another substrate) is the simplest and fastest method

Table 1. Similarity grouping and curli production of *Escherichia coli* isolated from cases of mastitis

Similarity group	Total isolates, n	Curli-positive isolates, n	Curli-negative isolates, n	Total cases, ¹ n	Curli-positive cases, n	Curli-negative cases, n
I	23	8	15	13	5	8
II	35	26	9	22	15	7
III	18	5	13	8	2	6
IV	23	19	4	12	10	2

¹From 1 to 4 isolates were obtained from each mastitis case.

Table 2. Daily milk yield of cows on d -5 and 7 of severe clinical mastitis, the curli phenotype, and similarity grouping of associated *Escherichia coli* isolates

Cow ID	Site ¹	Day -5 yield ²	Day 7 yield ³	Recovery ⁴	Curli phenotype	Similarity group
1083	B	20.00	32.73	1.64	+	IV
506	F	19.09	29.23	1.53	+	IV
12544	F	48.18	45.45	0.94	-	IV
5403	F	50.00	45.32	0.91	-	III
12364	F	34.55	31.32	0.91	-	III
1420	B	54.55	46.36	0.85	+	IV
1290	B	47.27	39.09	0.83	-	IV
1525	B	37.73	29.55	0.78	-	III
1080	B	50.00	34.55	0.69	+	IV
1135	B	36.36	24.55	0.68	+	IV
303	B	37.27	25.00	0.67	-/+ ⁵	III/IV ⁶
1450	B	41.36	26.36	0.64	-	III
1167	B	39.09	25.00	0.64	-	III
554	F	57.27	30.36	0.53	+	IV
541	B	45.45	23.64	0.52	+	III
1300 ⁷	F	20.00	6.18	0.31	+	IV
1129	B	36.82	— ⁸	—	+	IV
528	B	45.91	— ⁸	—	+	III
1343	B	47.73	— ⁸	—	—	IV

¹Origin of sample: B = New York, F = Colorado.²24-h milk yield (kg) 5 d before clinical mastitis event (d 0).³24-h milk yield (kg) 7 d after diagnosis of clinical mastitis event (d 0).⁴Ratio of d 7 to d -5 milk yield (d 0 = diagnosis of mastitis).⁵Isolate B303D0a and B303D0b = curli-negative; isolate B303D5c = curli-positive.⁶Isolate B303D0a and B303D0b = group III; isolate B303D5c = group IV.⁷Isolates from 2 quarters (left rear and right rear).⁸Cow died.

to determine if an *E. coli* isolate is capable of producing curli.

Curli expression was present in 59% of bovine cases, which is similar to the occurrence reported on a per-isolate basis by Olsen et al. (1989). In the 15 cases for which more than 1 isolate was cultured from the same cow, all isolates cultured from the same case displayed the same phenotype, with one exception. The most likely reason for this observation is that additional isolates cultured from the same case were either very closely related or identical clones. Another possibility is that curli-producing bacteria may have a selective advantage within the mammary environment of some cows, increasing the likelihood of culturing a curli-positive strain.

The *E. coli* cultured from cow B303 were the only isolates to have mixed curli phenotypes. The d 0 isolates were curli negative, whereas the isolate cultured on d 5 was positive. One possible explanation for this outcome is that the initial infecting population experienced a mutation or phase shift that allowed it to activate curli production during the clinical episode or that curli production was activated as an adaptive response (Jubelin et al., 2005). The genetic distance observed by ERIC-PCR between B303D0a and B303D0b (98% similarity), and B303D5c (52% similarity to B303D0a/b),

however, indicates that multiple, unrelated isolates may have been cultured from cow B303.

Indeed, genetically diverse isolates were cultured occasionally from the same cow. Sibling isolates in 7 cases clustered at <90%. In 4 of these cases, sibling isolates clustered at ≥80% similarity whereas isolates from 3 cows, cows B1450, F1300, and B303, clustered at <70% similarity. Cow B1450 had 3 isolates cultured from d 0 and 5 that clustered closely together (≥96% similarity), but also contained isolate B1450D0a that was only 60% similar to the others. Cow F1300, which was the only case in this study that presented mastitis in 2 quarters, had isolates from the right rear quarter that were very similar (96%) to each other, but were only about 70% similar to the isolates cultured from the left rear quarter, which were only 83% similar to each other. These data indicate that these cows were simultaneously infected with at least 2 genetically diverse *E. coli* strains. Mechanisms for the isolation of multiple *E. coli* strains include infection of the mammary gland by 2 or more strains, a separate infection that occurred after d 0, or contamination of the culture by an environmental *E. coli*. These strains could all be opportunistic invaders if the mastitis was the consequence of a systemic condition (such as stress, hormonal, or nutritional disorders) that caused temporary immunosuppression.

The ERIC-PCR did not differentiate curli-positive *E. coli* from those unable to produce curli in this study. Why such differences were not detectable by ERIC-PCR might be because the genetic capabilities required for curli expression are not located between ERIC elements or that they do not greatly alter the length of DNA (such as with the deletion of 1 base pair). In addition, curli production in these isolates may be entirely regulated by RNA, which was not examined, or the diversity of ERIC sequences and locations within the genome may overshadow any differences that contribute to curli production. The ERIC-PCR did allow limited differentiation of strains by origin of isolates. Isolates from cows in different sites were in general <90% similar, with the exception of 5 clusters of isolates from 13 cows from various origins, and which had ≥90% similarity (Figure 2).

The role of curli in the pathogenesis of bovine mastitis is unknown. When Todhunter et al. (1991) examined the outcome of experimental IMI with a curli-positive strain in cows vaccinated with either a curli-positive or a curli-negative strain of *E. coli*, all 12 cows developed clinical mastitis, regardless of curli expression of the vaccine strain. Our study evaluated the immediate effects of curli as measured by its effect on milk yields in a limited time frame. Curli expression did not affect the cows' recovery, when measured by the return to ≥75% of premastitis milk yield, the only available variable to evaluate severity of the mastitis case. Cows with severe clinical signs requiring aggressive supportive care may still be recovering by d 7, and impact of curli on mastitis may extend well beyond milk yields in that period.

Curli have been reported to enhance bacterial virulence, not only through enhanced adhesion, but also through the stimulation of inflammatory mediators, cytokines, and septic-related clinical signs (Nasr et al., 1996; Bian et al., 2000). Our study did not examine cows for evidence of expression of curli outside the mammary gland, such as for presence of antibodies to the curlin subunit, CsgA. Curli have been reported to facilitate cellular internalization of *E. coli*. Curli may play a role in chronic and recurrent mastitis by allowing some strains to reside in the mammary gland, serving as a reservoir for future infections. Possible areas of investigation to assess curli effect on bovine mastitis could include evaluation of the effect of curli on local and systemic clinical signs exhibited in mastitic cows, experimental mammary challenges with curli-positive and curli-negative *E. coli* strains, detection of CsgA antibodies in the serum of cows having severe or septic mastitis, and characterization of the role of curli in chronic, recurring mammary infections.

CONCLUSIONS

Curli expression was detected in 57% of *E. coli* isolated from cases of bovine mastitis in our study. This finding is very similar to previous reports of curli production among bovine mastitis and fecal isolates. Two genes that are used in curli formation and regulation, *csgA* and *csgD*, were present in all examined isolates, regardless of their curli phenotype. Curli-producing *E. coli* strains did not selectively cluster when fingerprinted by ERIC-PCR.

Generally, clinical severity of *E. coli* mastitis is more dependent on cow-related factors than on bacterial virulence. Curli production in *E. coli* isolated from clinical cases of mastitis in this study did not affect return to precase milk yield, and *E. coli* strains did not cluster based on case recovery when fingerprinted by ERIC-PCR. In addition, genetically diverse *E. coli* were recovered from clinically infected udders.

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REFERENCES

- Bian, Z., A. Brauner, Y. Li, and S. Normark. 2000. Expression of and cytokine activation by *Escherichia coli* curli fibers in human sepsis. *J. Infect. Dis.* 181:602–612.
- Bian, Z., Z. Q. Yan, G. K. Hansson, P. Thoren, and S. Normark. 2001. Activation of inducible nitric oxide synthase/nitric oxide by curli fibers leads to a fall in blood pressure during systemic *Escherichia coli* infection in mice. *J. Infect. Dis.* 183:612–619.
- Brody, J. R., and S. E. Kern. 2004. Sodium boric acid: A Tris-free, cooler conductive medium for DNA electrophoresis. *Biotechniques* 36:214–216.
- Cogan, T. A., F. Jorgensen, H. M. Lappin-Scott, C. E. Benson, M. J. Woodward, and T. J. Humphrey. 2004. Flagella and curli fimbriae are important for the growth of *Salmonella enterica* serovars in hen eggs. *Microbiology* 150:1063–1071.
- Collinson, S. K., L. Emody, K. H. Muller, T. J. Trust, and W. W. Kay. 1991. Purification and characterization of thin, aggregative fimbriae from *Salmonella enteritidis*. *J. Bacteriol.* 173:4773–4781.
- Cookson, A. L., W. A. Cooley, and M. J. Woodward. 2002. The role of type 1 and curli fimbriae of Shiga toxin-producing *Escherichia coli* in adherence to abiotic surfaces. *Int. J. Med. Microbiol.* 292:195–205.
- de Moura, A. C., K. Irino, and M. C. Vidotto. 2001. Genetic variability of avian *Escherichia coli* strains evaluated by enterobacterial repetitive intergenic consensus and repetitive extragenic palindromic polymerase chain reaction. *Avian Dis.* 45:173–181.

- Dopfer, D., H. W. Barkema, T. J. Lam, Y. H. Schukken, and W. Gaastra. 1999. Recurrent clinical mastitis caused by *Escherichia coli* in dairy cows. *J. Dairy Sci.* 82:80–85.
- Goldwater, P. N., and K. A. Bettelheim. 2002. Curliated *Escherichia coli*, soluble curlin and the sudden infant death syndrome (SIDS). *J. Med. Microbiol.* 51:1009–1012.
- Gophna, U., M. Barlev, R. Seijffers, T. A. Oelschläger, J. Hacker, and E. Z. Ron. 2001. Curli fibers mediate internalization of *Escherichia coli* by eukaryotic cells. *Infect. Immunol.* 69:2659–2665.
- Gophna, U., T. A. Oelschläger, J. Hacker, and E. Ron. 2002. Role of fibronectin in curli-mediated internalization. *FEMS Microbiol. Lett.* 212:55–58.
- Hammar, M., A. Arnqvist, Z. Bian, A. Olsen, and S. Normark. 1995. Expression of two *csg* operons is required for production of fibronectin- and Congo red-binding curli polymers in *Escherichia coli* K-12. *Mol. Microbiol.* 18:661–670.
- Hammar, M., Z. Bian, and S. Normark. 1996. Nucleator-dependent intercellular assembly of adhesive curli organelles in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 93:6562–6566.
- Hogan, J. S., R. N. González, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith. 1999. Testing procedures. Pages 205–217 in *Laboratory Handbook on Bovine Mastitis*. National Mastitis Council, Inc., Madison, WI.
- Jubelin, G., A. Vianney, C. Beloin, J. M. Ghigo, J. C. Lazzaroni, P. Lejeune, and C. Dorel. 2005. CpxR/OmpR interplay regulates curli gene expression in response to osmolarity in *Escherichia coli*. *J. Bacteriol.* 187:2038–2049.
- la Ragione, R. M., R. J. Collighan, and M. J. Woodward. 1999. Non-curling of *Escherichia coli* O78:K80 isolates associated with IS1 insertion in *csgB* and reduced persistence in poultry infection. *FEMS Microbiol. Lett.* 175:247–253.
- la Ragione, R. M., A. R. Sayers, and M. J. Woodward. 2000. The role of fimbriae and flagella in the colonization, invasion, and persistence of *Escherichia coli* O78:K80 in the day-old-chick model. *Epidemiol. Infect.* 124:351–363.
- Maurer, J., T. Brown, W. Steffens, and S. Thayer. 1998. The occurrence of ambient temperature-regulated adhesins, curli, and temperature-sensitive hemagglutinin Tsh among avian *Escherichia coli*. *Avian Dis.* 42:106–118.
- Meacham, K. J., L. Zhang, B. Foxman, R. J. Bauer, and C. F. Marrs. 2003. Evaluation of genotyping large numbers of *Escherichia coli* isolates by enterobacterial repetitive intergenic consensus-PCR. *J. Clin. Microbiol.* 41:5224–5226.
- Nasr, A. B., A. Olsen, U. Sjöbring, W. Müller-Esterl, and L. Björck. 1996. Assembly of human contact phase proteins and release of bradykinin at the surface of curli-expressing *Escherichia coli*. *Mol. Microbiol.* 20:927–935.
- Olsen, A., A. Arnqvist, M. Hammar, S. Sukupolvi, and S. Normark. 1993. The RpoS sigma factor relieves H-NS-mediated transcriptional repression of *csgA*, the subunit gene of fibronectin-binding curli in *Escherichia coli*. *Mol. Microbiol.* 7:523–536.
- Olsen, A., A. Jonsson, and S. Normark. 1989. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. *Nature* 338:652–655.
- Römling, U., Z. Bian, M. Hammar, W. D. Sierralta, and S. Normark. 1998. Curli fibers are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and regulation. *J. Bacteriol.* 180:722–731.
- Sjöbring, U., G. Pohl, and A. Olsen. 1994. Plasminogen, absorbed by *Escherichia coli* expressing curli or by *Salmonella enteritidis* expressing thin aggregative fimbriae, can be activated by simultaneously captured tissue-type plasminogen activator (t-PA). *Mol. Microbiol.* 14:443–452.
- Todhunter, D. A., K. L. Smith, J. S. Hogan, and L. Nelson. 1991. Intramammary challenge with *Escherichia coli* following immunization with a curli-producing *Escherichia coli*. *J. Dairy Sci.* 74:819–825.
- Uhlich, G. A., J. E. Keen, and R. O. Elder. 2001. Mutations in the *csgD* promoter associated with variations in curli expression in certain strains of *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 67:2367–2370.
- Uhlich, G. A., J. E. Keen, and R. O. Elder. 2002. Variations in the *csgD* promoter of *Escherichia coli* O157:H7 associated with increased virulence in mice and increased invasion of HEp-2 cells. *Infect. Immun.* 70:395–399.
- Uitenbroek, D. G. 1997. SISA-Binomial. <http://home.clara.net/sisa/binomial.htm> Accessed Jan. 1, 2005.
- Vidal, O., R. Longin, C. Prigent-Combaret, C. Dorel, M. Hooreman, and P. Lejeune. 1998. Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new *ompR* allele that increases curli expression. *J. Bacteriol.* 180:2442–2449.
- Zogaj, X., W. Bokranz, M. Nimtz, and U. Römling. 2003. Production of cellulose and curli fimbriae by members of the family *Enterobacteriaceae* isolated from the human gastrointestinal tract. *Infect. Immun.* 71:4151–4158.