Heterogeneity among Clinical Intestinal *Escherichia coli* Isolates upon Acquired Streptomycin Resistance

Lacey R. Lopez,[a] Claire M. Miller,[a] Joanna N. Jeyachandran,[a] Chuang Li,[a] Kenneth W. Simpson,[b] Janelle C. Arthur[a,c,d]

[a]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA
[b]Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA
[c]Center for Gastrointestinal Biology and Disease, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA
[d]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

Lacey R. Lopez and Claire M. Miller contributed equally to this article. Author order was determined by seniority.

ABSTRACT *Escherichia coli* isolates from inflammatory bowel disease (IBD) patients are often multidrug resistant, including to streptomycin. Streptomycin resistance (Str[R]) mutations can alter bacterial behavior, which may influence intestinal disease. We generated a spontaneous Str[R] strain of the intestinal adherent-invasive *E. coli* (AIEC) strain NC101. Whole-genome sequencing revealed a single missense mutation in *rpsL* that commonly confers Str[R], *rpsL*-K43N. Str[R] NC101 exhibited a striking loss of aggregation and significantly increased motility, behaviors that can impact host-microbe interactions. Behavioral changes were associated with reduced transcription of *csgA*, encoding the biofilm component curli, and increased transcription of *fliC*, encoding flagellin. Scanning electron microscopy (SEM) detailed morphologic changes consistent with the observed alterations in multicellular behavior. Because intestinal *E. coli* isolates exhibit remarkable strain-specific differences, we generated spontaneous Str[R] mutants of 10 clinical *E. coli* phylotype B2 strains from patients with IBD, colorectal cancer, and urinary tract infection. Out of these 10 Str[R] clinical strains, two had altered colony morphology on Congo red agar (suggesting changes in extracellular products), and three had significant changes in motility. These changes were not associated with a particular *rpsL* mutation nor with the presence of virulence genes encoding the inflammation-associated *E. coli* metabolites yersiniabactin or colibactin. We conclude that common mutations in *rpsL*, which confer Str[R], can differentially alter disease-associated phenotypes across intestinal *E. coli* strains. These findings highlight the heterogeneity among seemingly similar intestinal *E. coli* strains and reveal the need to carefully study the strain-specific effects of antibiotic resistance mutations, particularly when using these mutations during strain selection studies.

IMPORTANCE We demonstrate that Str[R], commonly acquired through a single point mutation in *rpsL* (a gene encoding part of the 30S bacterial ribosome), strikingly alters the morphology and behavior of a key intestinal AIEC strain, NC101. These changes include remarkably diminished aggregation and significantly increased motility, traits that are linked to AIEC-defining features and disease development. Phenotypic changes were heterogeneous among other Str[R] clinical *E. coli* strains, underscoring the need to evaluate the strain-specific effects of commonly acquired antibiotic resistance mutations. This is important, as the results of studies using mutant Str[R] *Enterobacteriaceae* strains (e.g., for cloning or *in vivo* selection) may be confounded beyond our demonstrated effects. Long term, these findings can help researchers better distinguish the contribution of specific *E. coli* traits to functional changes in the microbiota. Evaluating these strain-level differences could provide insight into the diversity of IBD symptoms and lead to improved therapies for microbiota-driven intestinal disorders.

Address correspondence to Janelle C. Arthur, janelle_arthur@med.unc.edu.

The authors declare no conflict of interest.

*E*scherichia coli isolates from IBD patients are frequently multidrug resistant, especially to aminoglycosides like streptomycin (1). Streptomycin resistance (Str[R]) can be acquired through spontaneous mutations in *rpsL*, which encodes the 30S ribosomal protein S12 (2). Str[R] is frequently caused by a missense mutation in *rpsL* that results in the substitution of lysine with asparagine at codon 42 or 43 (3). In this study, we reveal that the acquisition of Str[R] induced distinct behavioral and morphological changes in intestinal adherent-invasive *E. coli* (AIEC) strain NC101, similar to those observed in related pathogens (4–7). However, these alterations were not consistent among 10 additional, predominantly intestinal, clinical *E. coli* strains from the same B2 phylogroup as NC101. Inducing Str[R] is a common laboratory approach used during strain selection, and our results demonstrate that mutations in *rpsL* may not be as neutral as some may assume. Additionally, our studies highlight the importance of examining strain-specific behavioral traits that may alter host-microbe interactions with intestinal *E. coli*.

The *rpsL*-K43N Str[R] mutation drives behavioral and morphologic alterations in AIEC NC101. *E. coli* NC101 is a proinflammatory and procarcinogenic intestinal AIEC strain (6–9). To examine how Str[R] impacts the function of this broadly used strain, we generated a spontaneous Str[R] NC101 strain by passaging wild-type (WT) NC101 with increasing amounts of streptomycin and validated resistance via an MIC assay (Fig. 1A). Whole-genome sequencing revealed that Str[R] NC101 had only a single missense mutation in codon 43 of the *rpsL* gene, resulting in the substitution of lysine (K) with asparagine (N). We termed this mutation *rpsL*-K43N (Fig. 1B). Although there was no difference in the growth of WT and Str[R] NC101 cells in minimal defined medium (MM) (10) (Fig. 1C), Str[R] NC101 cells failed to aggregate in liquid culture, suggesting changes in extracellular product production (Fig. 1D and F).

As the extracellular product curli is an important component of *Enterobacteriaceae* biofilms, we assessed the expression of the curli biosynthesis gene *csgA*. Indeed, Str[R] NC101 expressed significantly less *csgA* (Fig. 1E). Because aggregation and swimming motility are often inversely activated, and altered motility can be a feature of AIEC (11), we measured the bacterial motility. As expected, Str[R] NC101 had significantly increased motility versus WT NC101 and the nonmotile control strain NC101 Δ*fliC* (Fig. 1G). The motility changes matched an upregulation of the flagellar biosynthesis gene *fliC* in Str[R] NC101 (Fig. 1H). Scanning electron microscopy (SEM) revealed changes to the cellular morphology between WT and Str[R] *E. coli* NC101, where WT cells had an abundance of fibers resembling curli and Str[R] NC101 predominately had flagella-appearing structures (11–13) (Fig. 1I). These data suggest that the *rpsL*-K43N mutation in NC101 confers Str[R] and, in MM, mediates a switch from the sessile to motile state for this important AIEC strain.

Acquired Str[R] has differential effects across clinical *E. coli* strains. *E. coli* isolates exhibit remarkable strain-specific differences in genome composition, behavior, and disease-inducing potential. Thus, we generated Str[R] isolates of 10 clinical *E. coli* strains, predominantly isolated from intestines and of the same B2 phylotype as NC101. None of these strains aggregated in MM; therefore, to qualitatively determine if Str[R] was associated with broad changes in extracellular products, we plated the strains on Congo red agar. WT NC101 displayed a red, dry, and rough (RDAR) morphology, previously shown to be dependent upon the production of curli and cellulose (14, 15). In contrast, Str[R] NC101 presented as red and smooth on Congo red, supporting observations in Fig. 1. Congo red also revealed that two strains, HM164 and HM229 (16), had similar changes as NC101 upon acquiring Str[R] (Fig. 2A).

We next performed motility assays on MM and LB soft agar, as nutrient availability can alter bacterial motility (17). Indeed, the motility of seven strains appeared different between MM and LB (Fig. 2B and C). More importantly, Str[R] significantly altered the motility of NC101 and three clinical strains (HM164, HM229, and UTI89), three were unaffected (CU08MY-1, HM44, and Nissle 1917), and the remaining four were nonmotile in the tested conditions (Fig. 1G and 2B to D).

Since there are several mutations in *rpsL* known to confer Str[R], we sequenced the *rpsL* gene of each WT and Str[R] strain. Sequencing revealed various *rpsL* mutations (Fig. 2D), suggesting that the observed changes in Congo red morphology and motility were not
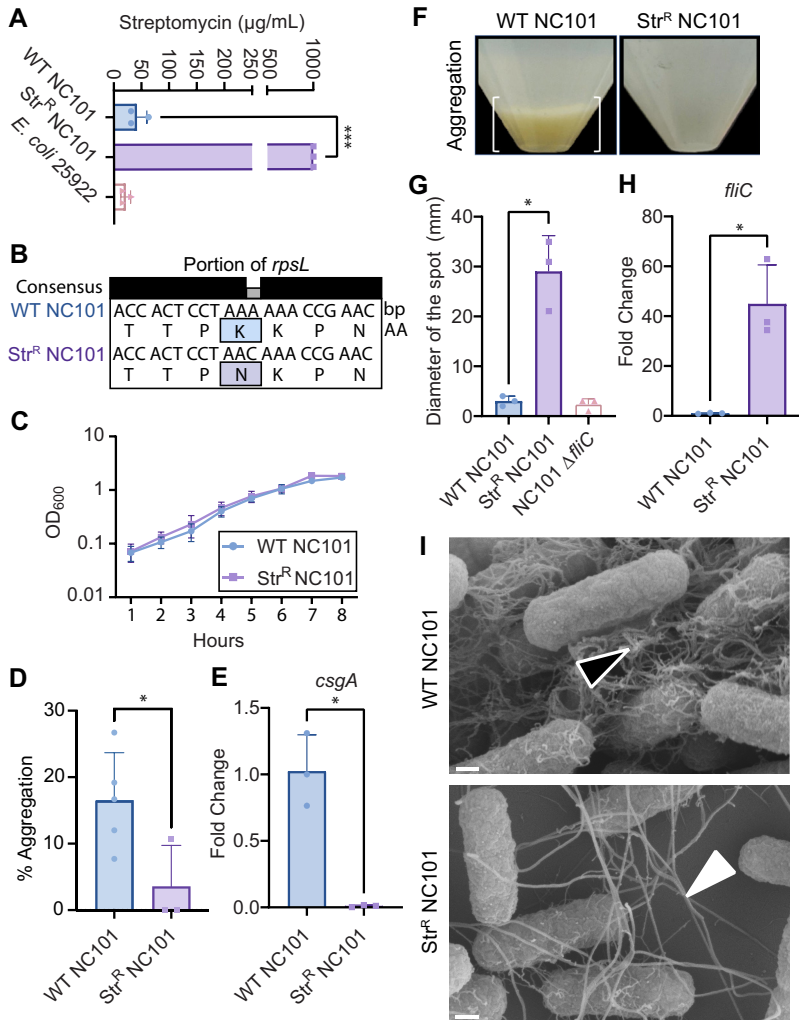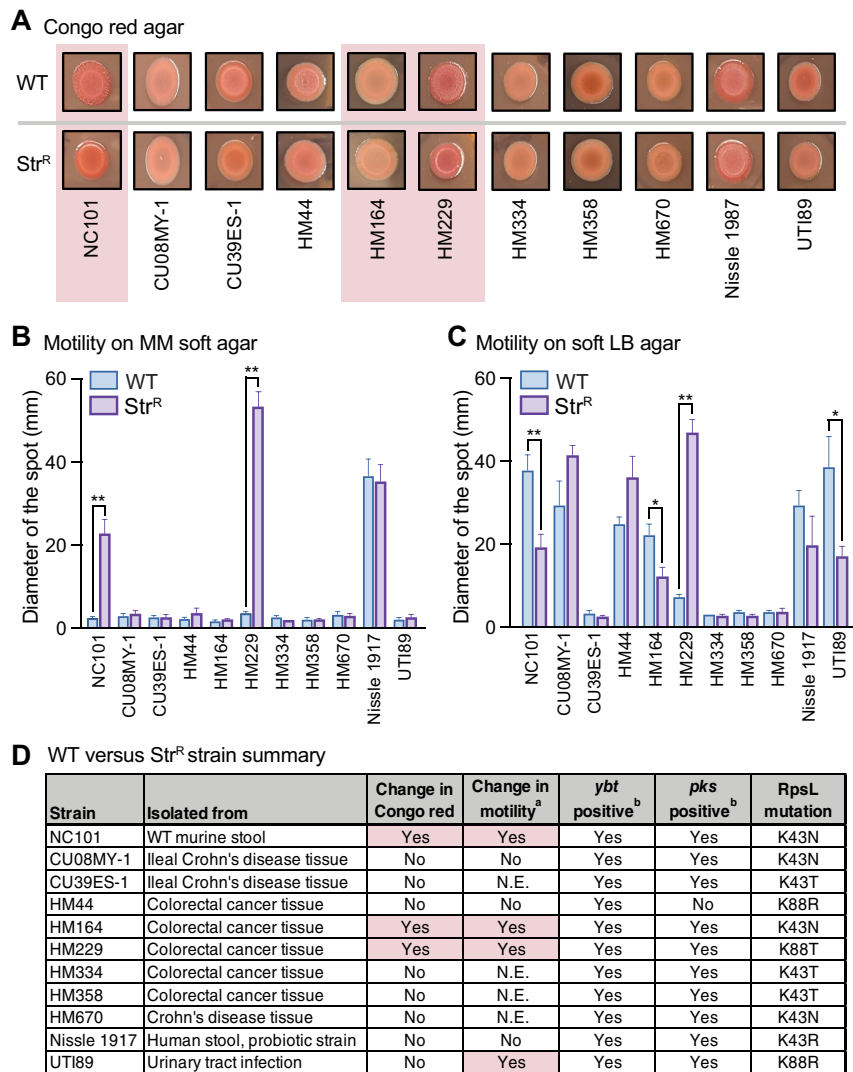
FIG 1 (A) MIC assays confirmed Str^R. (B) *rpsL* gene sequencing. (C) Growth curve in MM. (D) Aggregation quantification of panel F. (E) *csgA* expression, normalized to *uidA*, expressed as the fold change to WT NC101. (F) Aggregation (white brackets). (G) Motility swarms in MM. (H) *fliC* expression. (I) SEM ($\times$100,000; scale bar, 200 nm); the open triangle points to curlinacious fibers, and the closed triangle points to flagella-like products. All graphs represent the means $\pm$ standard error of the mean. *, $P < 0.05$; ***, $P < 0.001$; Welch's *t* test.

driven by a particular *rpsL* mutation. Furthermore, these mutations were not associated with the presence of *ybt* or *pks* genes, which encode disease-modulating metabolites yersiniabactin and colibactin (6, 8) (Fig. 2D). Taken together, we conclude that the effects of Str^R on *E. coli* morphology and behavior are heterogeneous and driven by strain-specific differences.

In summary, we reveal that the common *rpsL*-K43N mutation conferring Str^R is associated with dramatic changes in AIEC NC101 behavior, notably the production of extracellular products and motility. Extracellular products and motility impact proinflammatory behaviors, and hypermotile lineages of *E. coli* can be used to distinguish Crohn's disease patients from healthy controls (11, 15, 16, 18, 19). However, these morphological changes were inconsistent among NC101 and the 10 Str^R clinical *E. coli* strains. These findings highlight the importance of evaluating phenotypic changes across multiple *E. coli* strains that inhabit the intestinal niche, rather than generalizing conclusions based on the results from one strain, particularly in studies using antibiotic resistance mutations (11, 18, 20–22).

With the broad usage of antibiotics, there is a surge of antibiotic resistance across resident intestinal bacteria (1, 23, 24). One study found that an increased proportion of *Enterobacteriaceae* isolates from IBD patients were ciprofloxacin and rifaximin/rifampicin

A Congo red agar

B Motility on MM soft agar　　　**C** Motility on soft LB agar

D WT versus Str^R strain summary

Strain	Isolated from	Change in Congo red	Change in motility[a]	*ybt* positive[b]	*pks* positive[b]	RpsL mutation
NC101	WT murine stool	Yes	Yes	Yes	Yes	K43N
CU08MY-1	Ileal Crohn's disease tissue	No	No	Yes	Yes	K43N
CU39ES-1	Ileal Crohn's disease tissue	No	N.E.	Yes	Yes	K43T
HM44	Colorectal cancer tissue	No	No	Yes	No	K88R
HM164	Colorectal cancer tissue	Yes	Yes	Yes	Yes	K43N
HM229	Colorectal cancer tissue	Yes	Yes	Yes	Yes	K88T
HM334	Colorectal cancer tissue	No	N.E.	Yes	Yes	K43T
HM358	Colorectal cancer tissue	No	N.E.	Yes	Yes	K43T
HM670	Crohn's disease tissue	No	N.E.	Yes	Yes	K43N
Nissle 1917	Human stool, probiotic strain	No	No	Yes	Yes	K43R
UTI89	Urinary tract infection	No	Yes	Yes	Yes	K88R

[a]Motility change marked "yes" if present in either condition (MM or LB), N.E. = not evaluable
[b]Presence of biosynthetic gene cluster detected by PCR or sequencing, *ybt* = yersiniabactin and *pks* = colibactin

FIG 2 (A) Congo red morphology; the pink boxes indicate strains with phenotypic changes. (B and C) Motility swarms on MM (B) and LB (C) (mean ± standard error of the mean; *, $P < 0.05$; **, $P < 0.01$; *t* test, *n* = 3 to 7). (D) Strain table showing the phenotypes, virulence genes, and *rpsL* mutations; the pink boxes indicate key changes in WT versus Str^R strains.

resistant versus isolates from healthy individuals (1). In addition, similar to our study, changes in aggregation/motility have been associated with fluoroquinolone resistance and can enhance AIEC-defining characteristics (9, 11, 15, 25). Thus, it is important to evaluate disease-associated *E. coli* traits in response to these and other clinically important antibiotics. These findings may help explain the broad functional variation among genetically similar gut microbiota members and better inform treatment for IBD-related disorders.

Methods. (i) Bacterial strains. The NC101 and NC101 Str^R sequences are available at GenBank under accession numbers CP072787 and CP070227; the strains were generated as previously described (21). Strains CU39ES-1 and CU08MY-1 were from Kenneth W. Simpson at Cornell University (22); strains HM44, HM164, HM229, HM334, HM358, and HM670 were a gift from Barry Campbell at the University of Liverpool (16); and NC101 (6, 7), ATCC 25922 (26), and Nissle 1917 (26) were archived in the Arthur lab at the University of North Carolina (UNC), Chapel Hill. UTI89 was from Harry Mobley at the University of Michigan Medical School, and NC101 Δ*fliC* was from Melissa Ellermann at the University of South Carolina, Columbia.

(ii) Media and growth conditions. The strains were stored at −80°C with glycerol, and *E. coli* cells were grown at 37°C with shaking at 220 rpm in MM (containing nicotinic

acid, as NC101 is an auxotroph) (10). The aggregation and Congo red assays were previously described (15). For the motility assay, MM or LB soft agar plates (0.25% [wt/vol] agar) were inoculated with 1 μL overnight culture, and the motility swarm diameters were measured after 6 h (LB) or 8 h (MM) at 37°C. For the MIC assay, streptomycin was diluted 2-fold from 1,000 μg/mL in 200 μL/well MM and inoculated with 2 μL overnight bacterial culture. After 12 to 18 h at 37°C with shaking at 220 rpm, the MIC was recorded as the lowest concentration of streptomycin required to prevent *E. coli* growth (optical density at 600 nm [OD_{600}]). ATCC 25922 was a susceptible control strain.

(iii) Transcriptional analysis. Bacterial overnight cultures were back-diluted to an OD_{600} value of 0.05 in MM and grown at 37°C with shaking at 220 rpm for 8 h. Quantitative PCR (qPCR) was performed as previously described (26). Threshold cycle (C_T) values were normalized to *uidA*. The primers used were as follows: *uidA* forward (*uidA*-F), 5′-TCAACAG ACGCGTGGTTACAGTCT-3′; *uidA* reverse (*uidA*-R), 5′-TCCATCGCAGCGTAATGCTCTACA-3′; *csgA*-F, 5′-GGTAATAACAGCGGCCCGAA-3′; *csgA*-R, 5′-TGTCATCAGAACCTTGGCCC-3′; *fliC*-F, 5′-ACAACTACTGAGGATGCGGC-3′; and *fliC*-R, 5′-GCGGAATTTGTCGATCTGGC-3′.

(iv) SEM. SEM was performed at the Microscopy Services Lab (MSL) at UNC on bacteria pelleted from 8-h cultures in MM. The samples were imaged using a Zeiss Supra 25 FESEM device (5 kV, SE2 detector, 5 mm working distance, 30-μm aperture; Carl Zeiss SMT Inc., Peabody, MA).

(v) Targeted *rpsL* sequencing. A region of *rpsL* encoding amino acids 30 to 98 was amplified for Sanger sequencing (Genewiz/Azenta Life Sciences): F, 5′-AAACGTGGCGTATG TACTCG-3′, and R, 5′-TTGGAACGAGCCTGCTTAC-3′.

(vi) Rigor and reproducibility. All data are representative of at least three independent experiments.

REFERENCES

1. Dogan B, Scherl E, Bosworth B, Yantiss R, Altier C, McDonough PL, Jiang Z-D, DuPont HL, Garneau P, Harel J, Rishniw M, Simpson KW. 2013. Multidrug resistance is common in Escherichia coli associated with ileal Crohn's disease. Inflamm Bowel Dis 19:141–150. https://doi.org/10.1002/ibd.22971.

2. Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V. 2000. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature 407:340–348. https://doi.org/10.1038/35030019.

3. Paulander W, Maisnier-Patin S, Andersson DI. 2009. The fitness cost of streptomycin resistance depends on rpsL mutation, carbon source and RpoS (σS). Genetics 183:539–546. https://doi.org/10.1534/genetics.109.106104.

4. Chen C, Blumentritt CA, Curtis MM, Sperandio V, Torres AG, Dudley EG. 2013. Restrictive streptomycin resistance mutations decrease the formation of attaching and effacing lesions in Escherichia coli O157:H7 strains. Antimicrob Agents Chemother 57:4260–4266. https://doi.org/10.1128/AAC.00709-13.

5. Björkman J, Hughes D, Andersson DI. 1998. Virulence of antibiotic-resistant Salmonella typhimurium. Proc Natl Acad Sci U S A 95:3949–3953. https://doi.org/10.1073/pnas.95.7.3949.

6. Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan T-J, Campbell BJ, Abujamel T, Dogan B, Rogers AB, Rhodes JM, Stintzi A, Simpson KW, Hansen JJ, Keku TO, Fodor AA, Jobin C. 2012. Intestinal inflammation targets cancer-inducing activity of the microbiota. Science 338:120–123. https://doi.org/10.1126/science.1224820.

7. Kim SC, Tonkonogy SL, Albright CA, Tsang J, Balish EJ, Braun J, Huycke MM, Sartor RB. 2005. Variable phenotypes of enterocolitis in interleukin 10-deficient mice monoassociated with two different commensal bacteria. Gastroenterology 128:891–906. https://doi.org/10.1053/j.gastro.2005.02.009.

8. Ellermann M, Gharaibeh RZ, Fulbright L, Dogan B, Moore LN, Broberg CA, Lopez LR, Rothemich AM, Herzog JW, Rogala A, Gordon IO, Rieder F, Brouwer CR, Simpson KW, Jobin C, Sartor RB, Arthur JC. 2019. Yersiniabactin-producing adherent/invasive Escherichia coli promotes inflammation-associated fibrosis in gnotobiotic Il10$^{-/-}$ mice. Infect Immun 87:e00587-19. https://doi.org/10.1128/IAI.00587-19.

9. Darfeuille-Michaud A, Neut C, Barnich N, Lederman E, Martino PD, Desreumaux P, Gambiez L, Joly B, Cortot A, Colombel J-F. 1998. Presence of adherent Escherichia coli strains in ileal mucosa of patients with Crohn's disease. Gastroenterology 115:1405–1413. https://doi.org/10.1016/s0016-5085(98)70019-8.

10. Lopez LR, Barlogio CJ, Broberg CA, Wang J, Arthur JC. 2021. A nadA mutation confers nicotinic acid auxotrophy in pro-carcinogenic intestinal Escherichia coli NC101. Front Microbiol 12:670005. https://doi.org/10.3389/fmicb.2021.670005.

11. Elhenawy W, Tsai CN, Coombes BK. 2019. Host-specific adaptive diversification of Crohn's disease-associated adherent-invasive Escherichia coli. Cell Host Microbe 25:301–312.e5. https://doi.org/10.1016/j.chom.2018.12.010.

12. Evans ML, Chapman MR. 2014. Curli biogenesis: order out of disorder. Biochim Biophys Acta 1843:1551–1558. https://doi.org/10.1016/j.bbamcr.2013.09.010.

13. Abed SE, Ibnsouda SK, Latrache H, Hamadi F. 2012. Scanning electron microscopy (SEM) and environmental SEM: suitable tools for study of adhesion stage and biofilm formation. *In* Kazmiruk V (ed), Scanning electron microscopy. InTechOpen, London, UK. https://doi.org/10.5772/34990.

14. Römling U. 2005. Characterization of the rdar morphotype, a multicellular behaviour in Enterobacteriaceae. Cell Mol Life Sci 62:1234–1246. https://doi.org/10.1007/s00018-005-4557-x.

15. Ellermann M, Huh EY, Liu B, Carroll IM, Tamayo R, Sartor RB. 2015. Adherent-invasive Escherichia coli production of cellulose influences iron-induced

bacterial aggregation, phagocytosis, and induction of colitis. Infect Immun 83:4068–4080. https://doi.org/10.1128/IAI.00904-15.

16. Martin HM, Campbell BJ, Hart CA, Mpofu C, Nayar M, Singh R, Englyst H, Williams HF, Rhodes JM. 2004. Enhanced adherence and invasion in Crohn's disease and colon cancer. Gastroenterology 127:80–93. https://doi.org/10.1053/j.gastro.2004.03.054.

17. Wright KJ, Seed PC, Hultgren SJ. 2005. Uropathogenic Escherichia coli flagella aid in efficient urinary tract colonization. Infect Immun 73:7657–7668. https://doi.org/10.1128/IAI.73.11.7657-7668.2005.

18. Shaler CR, Elhenawy W, Coombes BK. 2019. The unique lifestyle of Crohn's disease-associated adherent invasive Escherichia coli. J Mol Biol 431:2970–2981. https://doi.org/10.1016/j.jmb.2019.04.023.

19. Martinez-Medina M, Naves P, Blanco J, Aldeguer X, Blanco JE, Blanco M, Ponte C, Soriano F, Darfeuille-Michaud A, Garcia-Gil LJ. 2009. Biofilm formation as a novel phenotypic feature of adherent-invasive Escherichia coli (AIEC). BMC Microbiol 9:202–202. https://doi.org/10.1186/1471-2180-9-202.

20. Viladomiu M, Metz ML, Lima SF, Jin W-B, Chou L, Bank JLC, Guo C-J, Diehl GE, Simpson KW, Scherl EJ, Longman RS, JRI Live Cell Bank. 2021. Adherent-invasive E. coli metabolism of propanediol in Crohn's disease regulates phagocytes to drive intestinal inflammation. Cell Host Microbe 29:607–619.e8. https://doi.org/10.1016/j.chom.2021.01.002.

21. Wang J, Bleich RM, Zarmer S, Zhang S, Dogan B, Simpson KW, Arthur JC. 2021. Long-read sequencing to interrogate strain-level variation among adherent-invasive Escherichia coli isolated from human intestinal tissue. PLoS One 16:e0259141. https://doi.org/10.1371/journal.pone.0259141.

22. Zhang S, Morgan XC, Dogan B, Martin F-P, Strickler SR, Oka A, Herzog J, Liu B, Dowd SE, Huttenhower C, Pichaud M, Dogan EI, Satsangi J, Longman R, Yantiss R, Mueller LA, Scherl E, Sartor RB, Simpson KW. 2022. Mucosal metabolites fuel the growth and virulence of E. coli linked to Crohn's disease. JCI Insight 7:e157013. https://doi.org/10.1172/jci.insight.157013.

23. Becattini S, Taur Y, Pamer EG. 2016. Antibiotic-induced changes in the intestinal microbiota and disease. Trends Mol Med 22:458–478. https://doi.org/10.1016/j.molmed.2016.04.003.

24. Kim S, Covington A, Pamer EG. 2017. The intestinal microbiota: antibiotics, colonization resistance, and enteric pathogens. Immunol Rev 279:90–105. https://doi.org/10.1111/imr.12563.

25. Dogan B, Zhang S, Kalla SE, Dogan EI, Guo C, Ang CR, Simpson KW. 2020. Molecular and phenotypic characterization of Escherichia coli associated with granulomatous colitis of boxer dogs. Antibiotics (Basel) 9:540. https://doi.org/10.3390/antibiotics9090540.

26. Sadecki PW, Balboa SJ, Lopez LR, Kedziora KM, Arthur JC, Hicks LM. 2021. Evolution of polymyxin resistance regulates colibactin production in Escherichia coli. ACS Chem Biol 16:1243–1254. https://doi.org/10.1021/acschembio.1c00322.