Invasion of epithelial cells are correlated with secretion of Biosurfactant via the type 3 secretion system (SST3) of *Shigella flexneri*

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Abstract

Biosurfactants are amphipathic molecules produced by many microorganisms, usually bacteria, fungi and yeasts. They possess the property of reducing the tension of the membrane interfaces. No studies have been conducted on *Shigella* species showing their involvement of biosurfactant like molecules (BLM) in pathogenicity. This study aims to show that environmental and clinical strains of *Shigella* are able to produce BLM by emulsifying gasoline and diesel fuels. Our study has shown that BLM are secreted in the extracellular medium with EI24 ranging from 80 to 100%. The secretion is depending on the type III secretion system (T3SS). We did show that *S. flexneri*, *S. boydii* and *S. sonnei* are able to interact with hydrophobic areas with respectively 17.64%, 21.42% and 22.22% of hydrophobicity. 100 mM Benzoic and 1.5mg/mL Salycilic acids have been inhibited T3SS and this totally stops the BLM secretion. *Pseudomonas aeruginosa* which has T3SS is able to produce 100% of BLM in the presence or in the absence of both T3SS inhibitors. The secreted BLM is extractable with an organic solvent such as chloroform and could entirely be considered like lipopeptide or polypeptidic compound. By

secreting BLM, *Shigella* is able to perform multicellular phenomena like "swarming" allowing to invade and disseminate inside epithelial cells.

Keywords: Shigella flexneri, Biosurfactant, Lipopeptide, Dissemination, Pathogens

Introduction

The ingestion of pathogenic and virulent microorganisms generally affecting peoples in both developed and developing countries [1]. *Shigella* is one of the Gram-negative bacterium belonging to *Enterobacteriaceae* family and is causative agent of bacillary dysentery or shigellosis [2]. The genus *Shigella* was the major pathogen bacteria associated with dysentery with attributable fraction to 63,8%, but also the second most common pathogen associated with watery diarrhoea with attributable fraction to 12,9% in sub-Saharan Africa and south Asia. Children under 5 years are the most affected. More and more shigellosis is a pathology that both towards neglected diseases but 164300 of death per years have been notified all over the world in 2010. Most deaths occur in sub-Saharan Africa and in south Asia [3-6]. This is include Republic of Congo and surprisingly no epidemiological studies have been conducted in this field. The genus Shigella includes four species (*S. flexneri*, *S. sonnei*, *S. dysenteriae* and *S. boydii*) [7]. 10 bacteria of *S. dysenteriae* type 1 and 100 to 180 bacteria of *S. flexneri* or *S. sonnei* are enough to produce symptomatic infection [8].

Shigella's pathogenicity is based on a virulence plasmid pWR100 in which the mxi-spa locus encodes the type three secretion system(T3SS) involved in effector production like IpaB, C and D (Translocator and Tip) to invade host cell [9-12]. A previous study in our laboratory that showed that *Shigella* sp. isolated from Brazzaville wastewater were able to emulsify hydrocarbon from gasoline and/or diesel fuel [13]. Sachin et al. found the same profile of hydrocrabon emulsification with *Shigella* strain [14]. According to amphipathic features, biosurfactants display a variety of surface activities, which explain their application in several fields related with emulsification, foaming, detergency, wetting, dispersion, pathogenicity and

49 solubilisation of hydrophobic compounds[15, 16]. Biosurfactants are produced from
50 microorganisms like *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans*, and
51 *Acinetobacter calcoaceticus*. Rhamnolipids, sophorolipids, mannosylerithritol lipids, surfactin,
52 and emulsan are well known and documented in terms of biotechnological applications [16-
53 18].
54 *Shigella* pathogenicity mechanisms have been mostly studied using S. flexneri M90T as a
55 model. In this study, we need to demonstrate that *Shigella* strains produce a biosurfactant in
56 extracellular medium. Inner and outer membrane encompass numbers of secretion systems. In
57 this way, this work aims to study the involvement of BLM via the type three secretion system
58 (T3SS) pathways. In addition this work will assess the approvals that *Shigella* could use the
59 BLM to promote the invasion and the dissemination inside epithelial cells.
60

61 ## Material and methods

62 ### Strains and Culture Conditions

63 Four (4) *Shigella* strains were kindly provided and collected from laboratory of Molecular
64 Bacteriology (Free University of Brussels). This is include *S. flexneri* M90T, *S. flexneri spa40*-,
65 *S. sonnei* and *S. boydii*. Three (3) pure culture strains were isolated from patients in Brazzaville
66 University and Hospital Center (CHU-B) in 2018. These were provided by the Bacteriology
67 Laboratory this hospital. Thirty (30) *Shigella* sp. strains were isolated in environmental
68 wastewater of Brazzaville using decimal dillution in SS medium. *P. aeruginosa* [19] and E. coli
69 Top10 were used as controls in this study. The strains were spread on the plates containing LB
70 medium with Congo red with 100 µg/mL streptomycin for 24 hours at 37 ° C for wild type and
71 50 µg/mL for *spa40* mutant.

72 ### Emulsification index (EI24) assay

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An overnight of 5mL of bacterial culture have been done. The emulsification index (EI24) was calculated as an indicator for BLM as previously demonstrated [13]. The medium was adjusted to pH 7.2 and supplemented with gasoline or diesel fuel (1 mL for 300 mL of medium). The EI24 was investigated by adding fuels with LB medium in 1:1 ratio (v/v). The solution was vortexed for 5 min and incubated for 24 h at 37°C. The emulsification rate was calculated through the height of the emulsion layer. In addition, EI24 was determined for gasoline and diesel fuel hydrocarbons. All the experiments were performed in triplicates, EI24 = height of emulsion layer/total height of solution × 100.

Bacterial swarming assays.

Swarming was studied for all *Shigella* strain used in this study. Using plate assays containing 0.5% noble agar and LB medium with 0.5% dextrose. The mixture was sterilized at 121°C, during 15 min. After sterilization, the medium was supplemented with adequate antibiotics including streptomycin 100µg/mL for wild type and kanamycin 50 µg/mL for the Shigella flexneri spa40 mutant. Approximately 6 h after pouring the plates, bacteria were inoculated and spread by using a sterilized platinum wire with log-phase cells ([OD600] 0.6) grown in their respective media used for the swarming experiments. Swarming plates that were imaged only for their comparative end point swarming development were incubated at 30°C for 24 h prior to imaging [20].

Bacterial Adhesion assay

The adhesion of bacteria to hydrophobic surface was evaluated according to the method described by Rosenberg [21]. The hydrophobicity was evaluated according to the following formula %H=A0-A/A0*100 with A_0: OD before the mix; A: OD after vortexing of aqueous phase.

Induction assay by using Congo Red

Shigella sp. have been cultivated in 5 mL of the final volume. 1 mL of overnight culture was fuged and 500 µL of sterile PBS and 10 µl of Congo red (10 mg / ml) have been gently added and mixed with the pellet by avoiding to break cells. Samples were incubated at 37 ° C with stirring. After 30 minutes of incubation, samples were centrifuged at 15.000 rpm for 15 minutes [11] at room temperature. Supernatants were gently recovered and mixture with gasoline or diesel fuels. The emulsification Index (EI24) have been determined.

Extraction of Biosurfactant like molecule

Three methods have been used to extract biosurfactant.

HCL and Ethanol precipitation: This method was described by Vater [22]. An overnight culture has been centrifuged at 13,000 × g for 15 minutes. Once the supernatant was collected, HCl 1N and 90° ethanol were added to the supernatant. Precipitates have been generated by incubating samples at 4 ° C in overnight. Mixtures were fuged at 13000 g for 15 minutes to obtain granules. The granules obtained were tested with EI24 to evaluate the ability to emulsify the hydrocarbons.

*Ammonium sulfate precipitation test***:** An overnight culture has been fuged at 13,000 rpm for 15 minutes to separate supernatant and pellet. Then 15 mL of supernatant were mixed with ammonium sulfate (80%) for 15 minutes. And finally this has been incubated with shaking overnight. The mixture has been fuged at 6000 rpm for 30 minutes at room temperature. Pelet has been hommogenized by using PBS buffer. The emulsification activity has been assessed.

Biosurfactant Extraction using Chloroform: The 24h culture was strictly centrifuged at 15,000 g for 15 minutes to avoid any residual bacteria. One volume of supernatant was added with an equal volume of chloroform (v/v). The mixture was strongly agitated by a vortex. After centrifugation at 6000 rpm for 10 min, the non-aqueous phase is recovered. The solvent was

allowed to evaporate completely only without heating above 40˚C. The residue is dissolved in a PBS buffer. The emulsification activity is tested by mixing with gasoline or diesel fuel in comparison with the supernatant at the start point. The emulsification Index (EI24) have been determined.

Effect of Benzoic Acid and Salycilic acid on Biosurfactant secretion

Viability of *Shigella* strains have been first evaluated with different concentration of benzoic acid and salycilic acid. *S. flexneri* M90T was grown in Luria-Bertani broth (LB) in the presence of various concentrations of benzoic acid (50 mM, 100 mM, 250 mM and 500mM) and salycilic acid (1.5 mg/mL, 3 mg/mL, 6.25 mg/mL and 12.5 mg/mL). After that, all Shigella strain was grown in Luria-Bertani broth (LB) added with an adequate concentration of benzoic acid or salycilic acid at 37°C, during 24 hours. All supernatants were fuged and the secretion biosurfactants was assessed by using emulsification assay (EI24).

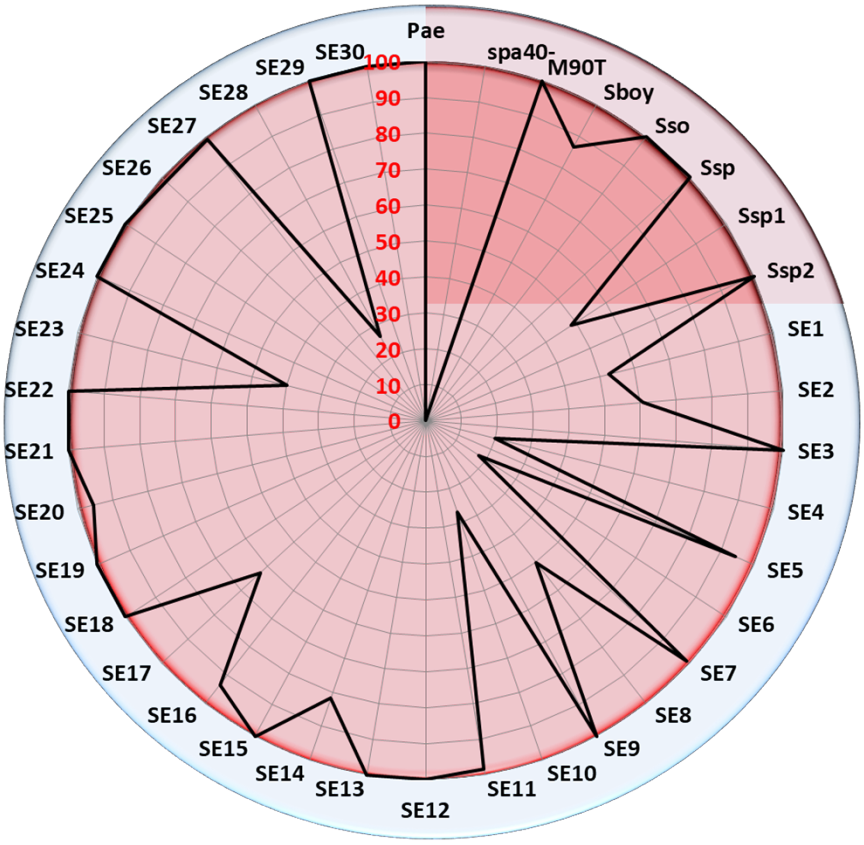
Statistical analysis

GraphPad Prism 7 and Excel software were used for analysis. The data represent the arithmetical averages of at least three replicates. Data were expressed as mean ± SD and Student's test was used to determine statistical differences between strains and $p < 0.05$ was considered as significant. Principal Component Analysis (PCA) was used to investigate possible correlations between *Shigella* and emulsification index (EI24). Prior to ordination, percentage of emulsification activities data were transformed to better meet the assumptions of normality [23] using ln (x+1). All analysis was performed using CANOCO (Canonical Community Ordination, version 4.5) [24].

148 ## Results

149 ### Screening for biosurfactant production

150 In order to carry out our research, we first assess if *Shigella* strains are able to produce BLM in
151 extracellular medium. As results Figure 1 showed that environmental strains and clinical strains
152 are able to secrete BLM by showing emulsification percentages ranging between 15% to 100%
153 (Figure 1A). *S. flex*neri spa40- was not able to produce BLM compare with *Pseudomonas*
154 *aeruginosa* used as positive control. The way of strains to produce BLM is shown in figure 1B
155 all strains are not represented (Figure 1B).



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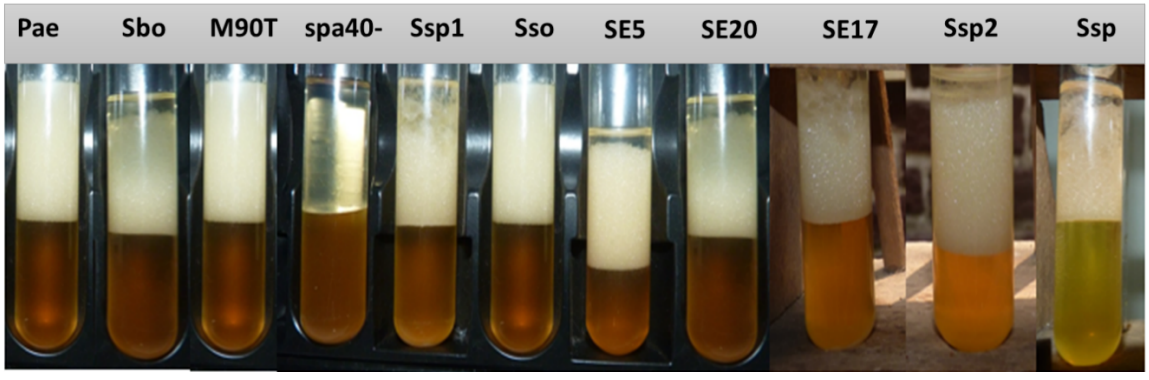

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Figure 1. A. Emulsification index percentage of all *Shigella* strains used in this study after 24 hours. Pae: *P. aeruginosa* used as positive control; M90T: *Shigella flexneri* 5a strain M90T; *spa40-*: *S. flexneri* spa40 mutant; Sbo: *S. boydii*; *Sso*: *S. sonnei*; *Ssp, Ssp1, Ssp2*: *Shigella* sp. from clinical strains; SE1…30: *Shigella* sp. from environmental strains. B. **Emulsification index appearance of some *Shigella* strains.**

EI24 of Some strains are ranging from 80 to 100%. This is included *Shigella flexeneri* M90T, *Shigella boydii* (Sbo), *Shigella sonnei* (Sso), *Shigella* sp (Ssp), Ssp2, SE3, SE5, SE9, SE11, SE12, SE13, SE14, SE15, SE16, SE18, SE20, SE21, SE22, SEI24, SE25, SE2626, SE27, SE29 and SE30 (Figure 2). The positive control has been found in this rate. SE1, SE8, SE23 and Ssp1 are ranging between 40 and 60%. SE4, SE10 and SE28 are between 20 and 40%. SE17 and SE2 from 60 to 80% and Shigella flexneri spa40 mutant (spa40-) is not able to produce biosurfactant and SE6 is about 17% ranging from 0 to 20% (Figure 2).

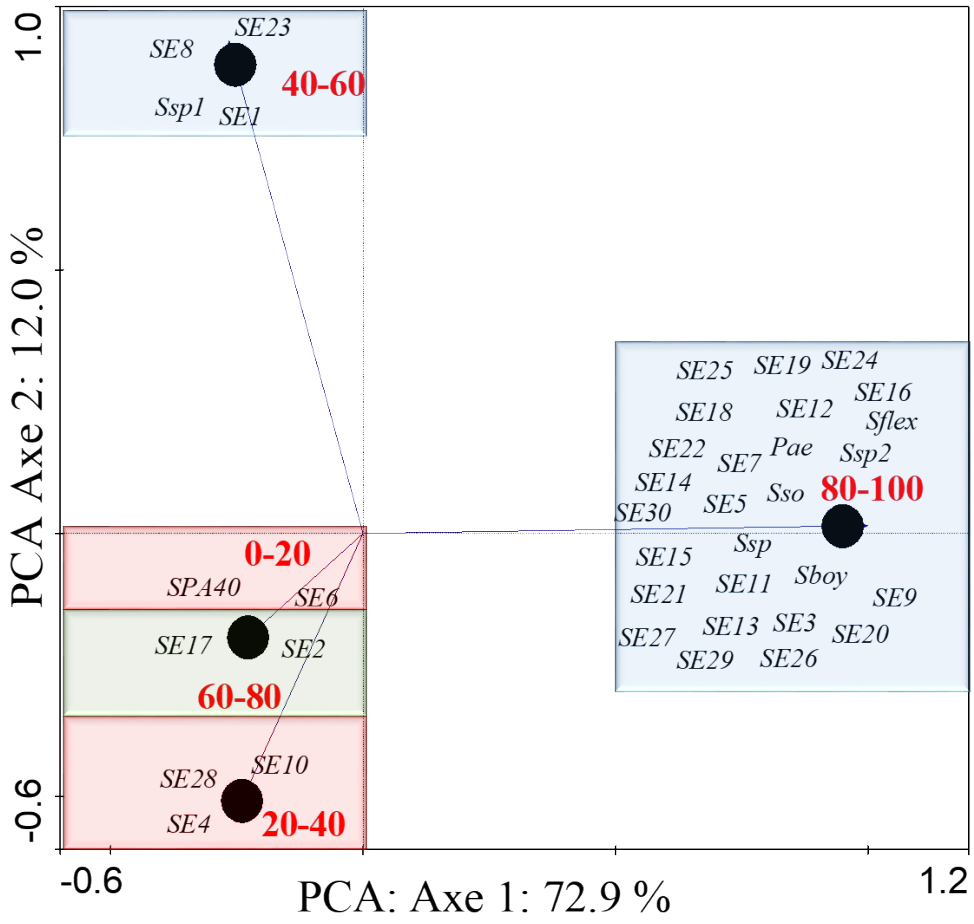


Figure 2: PCA of *Shigella* strains basing on emulsification index (EI24). Pae: P. aeruginosa used as positive control; S. flex: *S. flexneri* M90T; S. flex-: *S. flexneri spa40* mutant ; Sboy: *S. boydii*; S. so: *S. sonnei*; Ssp 1, 2: Shigella sp clinical strain; S. E1…30: *Shigella* sp environmental strain.

The ability of Shigella strain in swarming test

Swarming is induced by the production of BLM. In order to demonstrate how *Shigella* can disseminate in epithelial cell, we first investigated if all *Shigella* strains used in this study were able to swarm by using (0.5%) LB medium + 0.5% dextrose. As result *S. flexneri* M90T, *S. sonnei* and *S. boydii* were able to spread and swarm. *spa40*- was not able to swarm (Figure 3). Some examples of the swarming profile of some *Shigella* strain after 24 hours are illustrated. We found that *S. flexneri spa40*- cannot swarm and *S. sonnei* have a particular swarming profile than other *Shigella* strain used in this study.

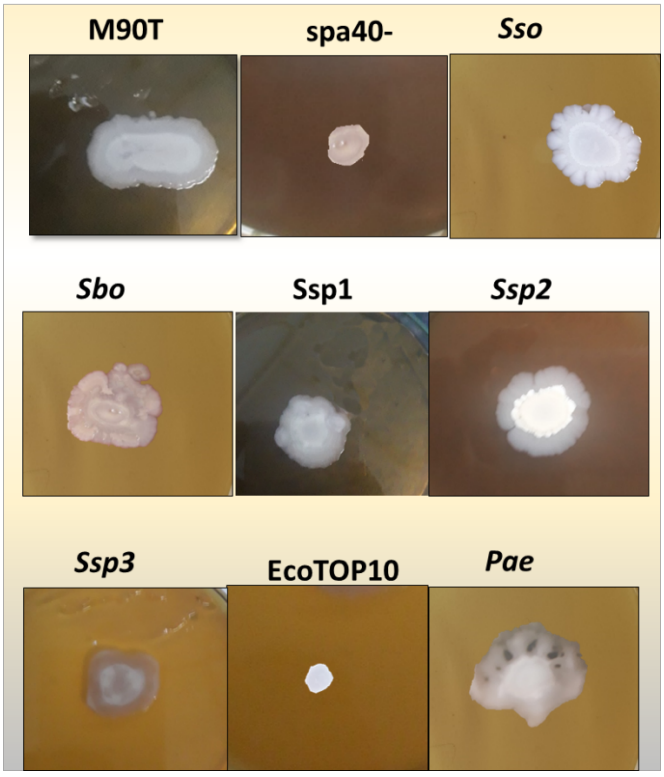
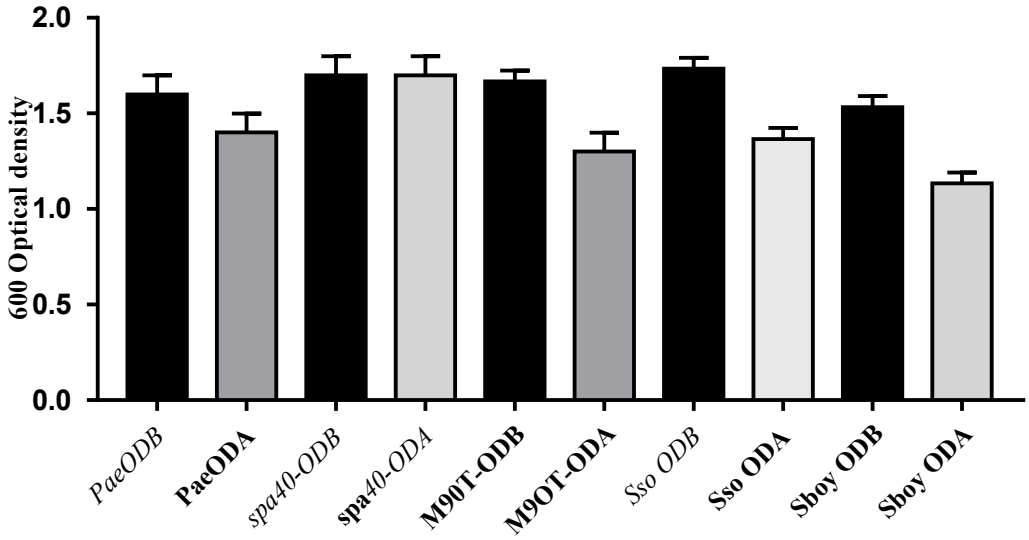


Figure 3 : Swarming profile of *Shigella strains.* M90T*: Shigella flexneri* 5a strain M90T. Sbo: *S. boydii*) ; Sso: *S. sonnei* ; *spa40-*: *S. flexneri* 5a *spa40-* ; Ssp1, 2, 3: *Shigella* sp. Pae: P. aeruginosa used as positive control and E. coli-Top10 used as negative control.

Bacterial Adhesion To Hydrocarbon (BATH)

To highlight the production of BLM by *Shigella* strains to induce interaction with hydrophobic areas, we performed analysis by evaluation of the ability to interact with hydrocarbon. The Figure 4 shows the bacteria adhesion profile of some *Shigella* strains. As results only *S. flexneri* Spa40 mutant does not interact with hydrocarbon area (Figure 4A). *S. flexneri*, *S. boydii* and *S. sonnei* are positive with BATH techniques including a percentage of hydrophobicity of 17.64%, 21.42% and 22.22% respectively (Figure 4B).



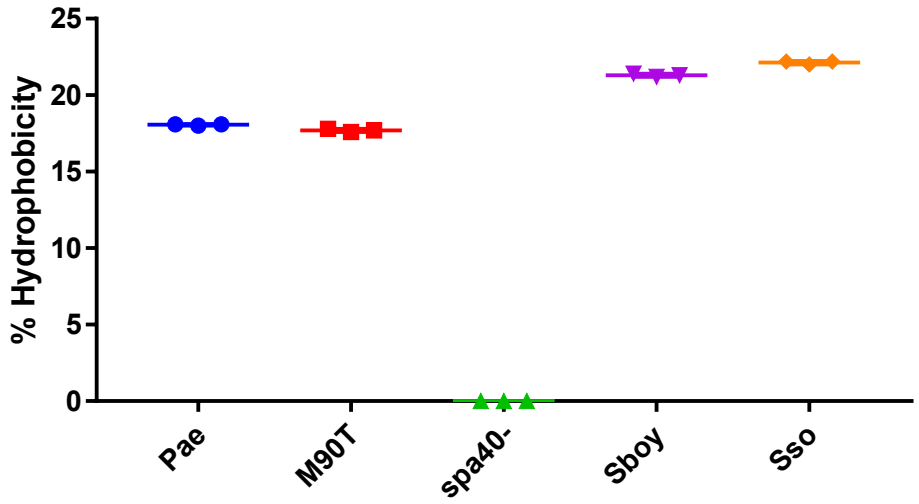
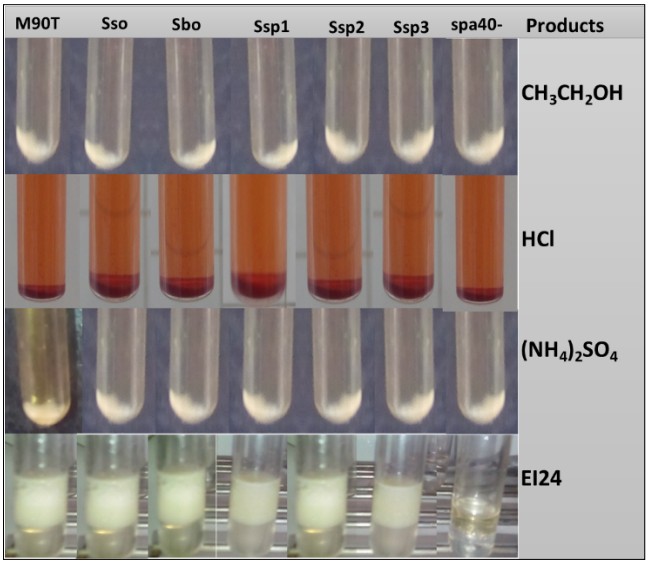


Figure 4: A. ***Shigella*'s adhesion to hydrocarbon phase of some strains used in this study. ODB: optical density before vortexing. ODA: optical density after vortexing. M90T: *Shigella flexneri* 5a strain *M90T*. *Sbo:* S. boydii*) ; Sso: S. sonnei ;* spa40-*:* S. flexneri *5a* spa40-. Pae: *P. aeruginosa* used as positive control. B. Percentage hydrophobicity of *Shigella* strains.**

Screening of biosurfactant secreted by *Shigella* sp.

To highlight the nature of the BLM secreted by *Shigella* strains used in this study. Cultures of *Shigella* strains whose supernatant emulsified hydrocarbons (gazoline or diesel fuel), have been used to identify the type of Biosurfactant like molecules. Precipitation on hydrochloric acid, ammonium sulphate and ethanol have been done. All strains showed a precipitate at the bottom of the tube. The emulsification index after precipitation has been carried on (EI24). Only precipitate profile performed from the *S. flexneri spa40-* supernatant did not emulsify the gas oil and/or diesel fuel. *S. flexneri, S. sonnei, S.* boydii and three Shigella sp. have 100 % of EI24 (**Figure 5**).



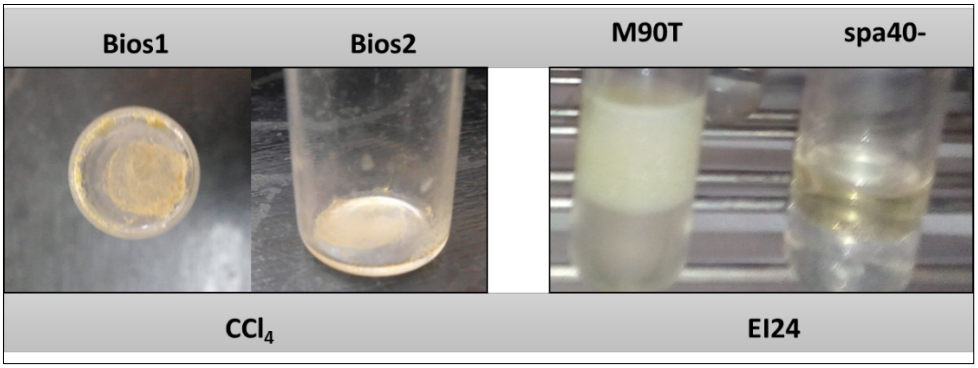
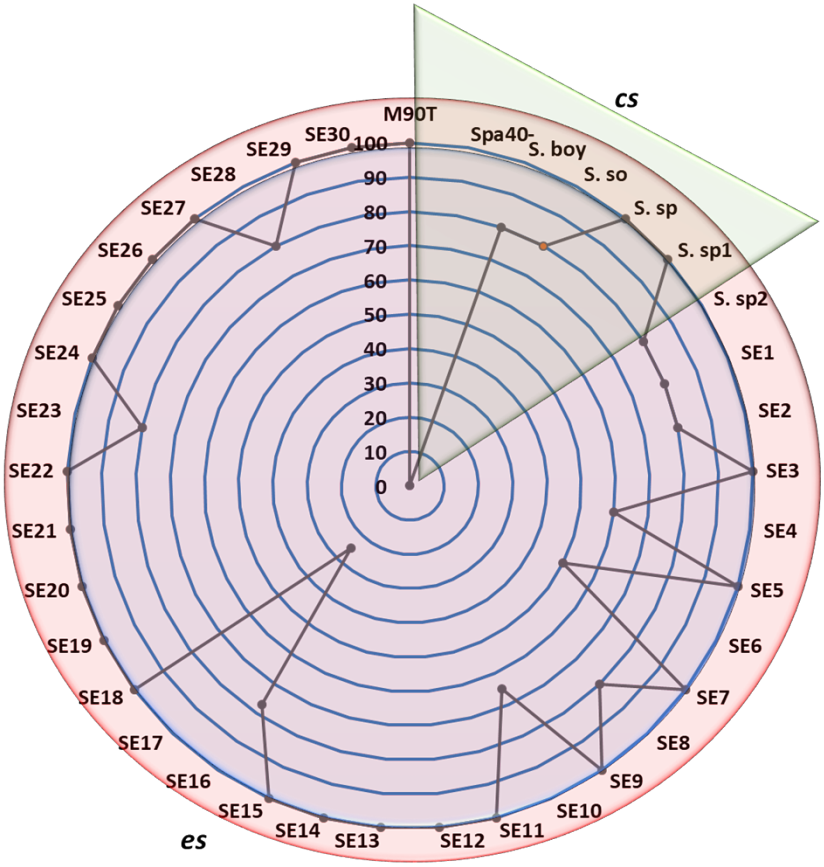


Figure 5: BLM purified from *Shigella Species*. TOP: Profile obtained after precipitation with ethanol (CH3CH2OH), Hydrochlorid acid (HCl) and ammonium sulfate ((NH4)2SO4). EI24: emulsification index for all strains (*S. flexneri* M90T, *S. sonnei*, S. *boydii*, *Shigella* sp.: Ssp1, 2, and 3. Down: left: residues obtained after evaporation of Chloroform (CCl4). Right: Emulsification index (EI24) for the extratable biosurfactant like molecule. Bios1 and Bios2: Biosurfactant like molecule residues.

Strains with known hydrocarbon emulsification ability were selected from an organic solvent like chloroform using biosurfactant extraction assay. Biosurfactant could be extracted after evaporation of chloroform at 40 ° C from *S. flexneri* M90T. Nothing was obtained from *spa40-* The extract after evaporation, suspended in PBS, was able to emulsify gasoline or gas oil with 100 % of EI24.

The BLM is secreted by Type Three Secretion System (T3SS)

Clinical strains including *S. flexneri* M90T, *S. sonnei*, *S. boydii*, three *Shigella* sp. and 30 environmental strains including SE1 to SE30 were cultivated to induce the secretion of effector on Congo red induction. As results *Shigella* species have been found to secrete BLM on Congo red induction conditions with EI24 ranging from 80 to 100% . The mutant *S. flexneri* spa40- did not emulsify the gasoline and /or diesel fuel in the presence of Congo induction with 0% of EI24 (Figure 6A). Emulsification after Congo red type 3 secretion system of *Shigella* strains appearance are illustrated in Figure 6 down.



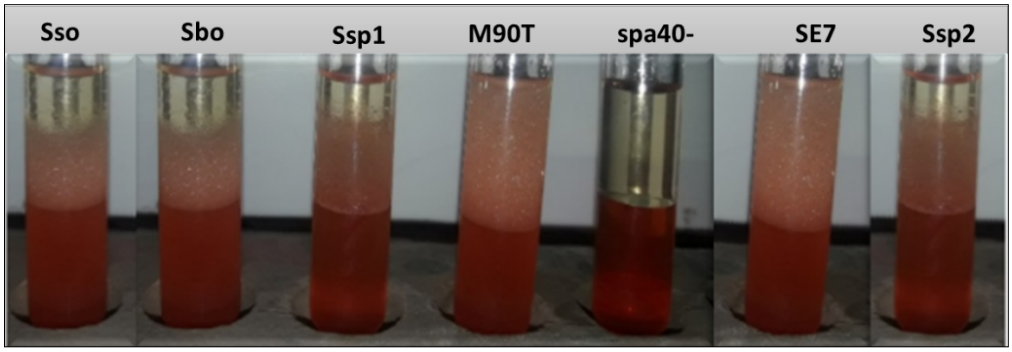
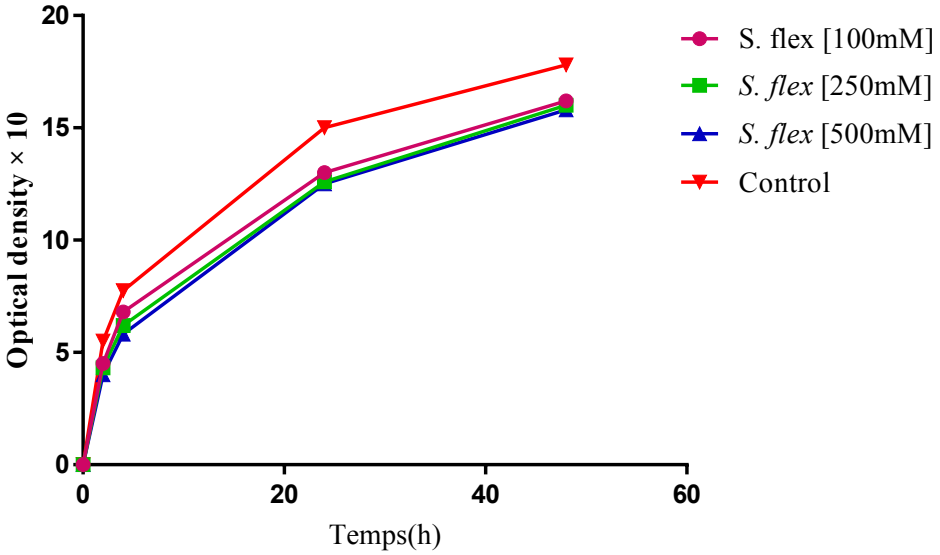


Figure 6. **Emulsification index after Congo red induction**. UP. **Pae: *P. aeruginosa* used as positive control; M90T: *Shigella flexneri* 5a strain M90T; *spa40-*: *S. flexneri spa40* mutant; Sbo: *S. boydii*; *Sso*: *S. sonnei*; *Ssp, Ssp1, Ssp2*: *Shigella* sp. from clinical strains; SE1…30: *Shigella* sp. from environmental strains. Down**. **Emulsification index appearance of some *Shigella* strains.**

Effect of Benzoic Acid and Salycilic acid on Biosurfactant production

All *Shigella* strains were grown in Luria-Bertani broth (LB) in the presence of random concentrations of benzoic acid and salycilic acid (**Figure 7**). We examined growth at the various concentration of benzoic acid including 50 mM, 100 mM, 250 mM and 500 mM. As far as salycilic acid is concerned 1.5 mg/mL, 3 mg/mL, 6.25 mg/mL and 12.5 mg/mL were randomly selected. All *Shigella* strain grew normally within the physiological range of benzoic acid as determined by CFU per milliliter, but growth was significantly interesting at 100 mM benzoic acid and 1.5 mg/mL for salycilic acid (Figure 7).



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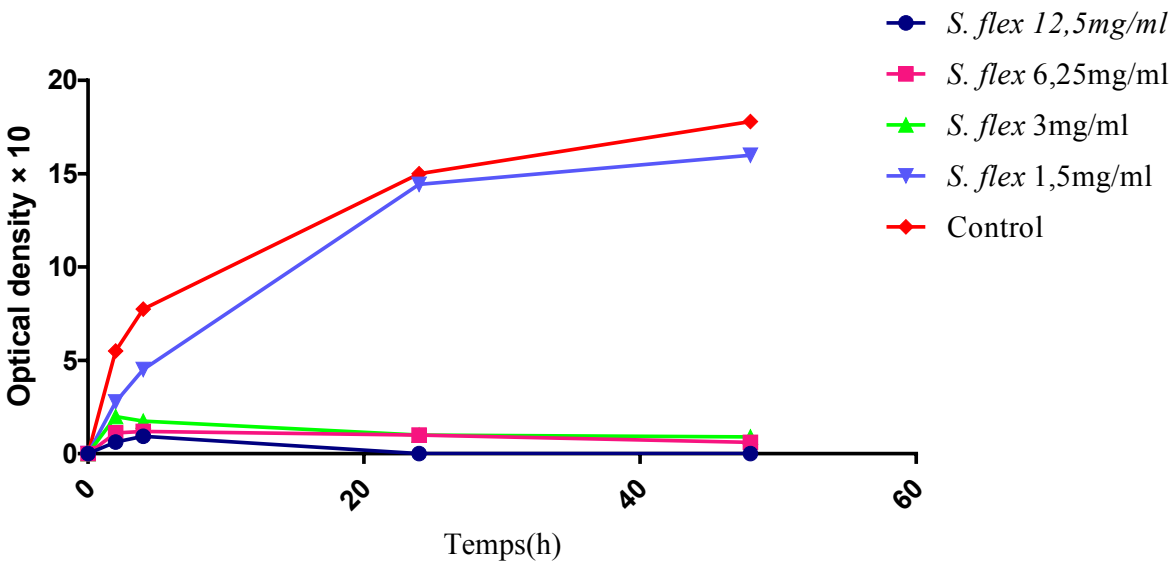

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254 **Figure 7. Growth curve analysis of all *Shigella* flexneri M90T used in this study on the**

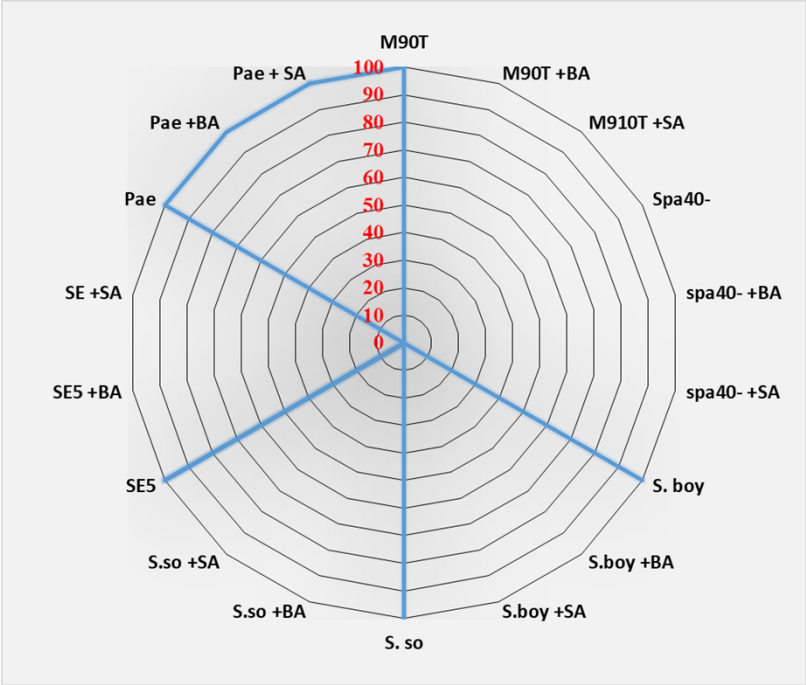
255 **presence of benzoic acid (TOP) and salycilic acid (Down).**

256 To highlight the role of T3SS on the secretion of BLM we assessed the effect of benzoic and

257 salycilic acids to inhibit the biosurfactant production. Bacteria were previously incubating

258 with100 mM benzoic acid (BA) and 1.5 mg/ml salycilic acid (SA), we showed that *S. flexneri*

M90T, *S. sonnei, S. boydii* and SE5 were not able to produce BLM with an emulsification index 0% EI24. This is easily showed that all *Shigella* strains do not emulsify anymore gasoline or diesel fuel with benzoic acid or salycilic acid (Figure 8 up). Strains are able to emulsify gasoline or diesel fuel without benzoic acid or salycilic acid (Figure 8down). The appearance are illustrated in Figure 8 down. *P. aeruginosa* has been used as positive control since the T3SS is widely conserved in the most gram negative bacteria, surprisingly *P. aeruginosa* was able to produce 100% BLM in the presence or in the absence of BA and SA. It is worthy noticed that *spa40-* was not able to produce BLM neither in the presence nor in the absence as up mentionned (Figure 8).



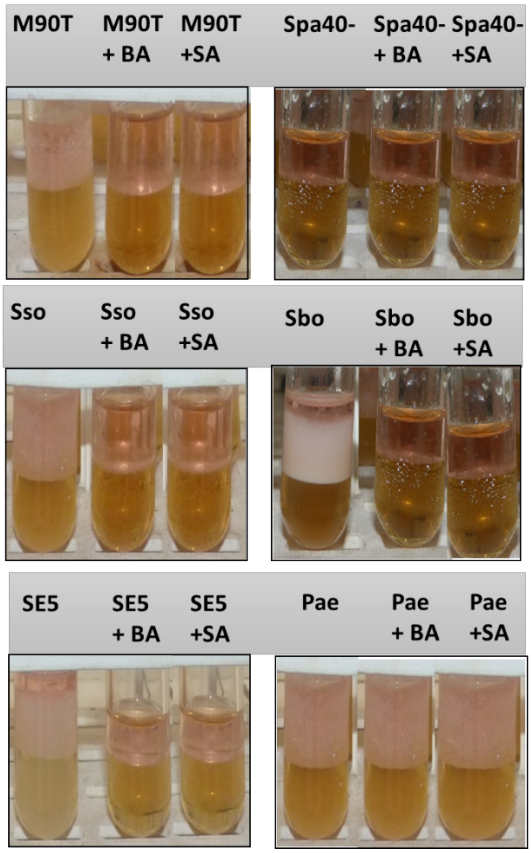


Figure 8: **(a). Gasoline emulsifying activity of some *Shigella* strains used in this study with and without benzoic acid (BA) and salycilic acid (SA). M90T: *S. flexneri* strain M90T. spa40-: *S. flexneri spa40* mutant, *Sso* : *S. sonnei*; *Sbo*: *S. boydii*; *S.E*: *Shigella* sp (environmental strain) and Pae: *P. aeruginosa* (b). Gasoline emulsifying activity appearances of some *Shigella* strain tested with and without benzoic acid or Salycilic acid.**

Discussion

This work was conducted with the prime aim to contribute to the understanding of the *S. flexneri* 5a M90T epithelial cell invasion mechanisms. *Shigella* strains had been collected from the environmental areas, hospital or laboratory. All strains had the ability to produce BLM during growth in extracellular medium, and the production is strictly depending on T3SS pathway.

282 This result shows very clearly that these molecules are secreted in the extracellular medium as
283 described by Usman and al. [25]. *spa40* mutant which has no T3SS cannot secrete BLM.
284 Several studies have been demonstrated the role of T3SS in the secretion of numbers of effector
285 proteins involved in invasion and dissemination [26-33].

286 The emulsification index is a direct method for demonstrating the ability of strains to produce
287 biosurfactants or not [13]. Those molecules have been known to form emulsions between two
288 immiscible liquids [34, 35]. Experiments carried out from the acellular supernatant showed that
289 *S. flexneri* 5a M90T as well as *S. boydii*, *S. sonnei* and other *Shigella* sp. are able to emulsifying
290 gasoline and diesel fuels with EI24 ranging from 80 to 100%. Gram negative bacteria are well
291 documented to overcome this phenomenon. This is include *P. aeruginosa* [34, 35], *Salmonella*
292 *enteridis* [36], *Acinetobacter* sp. [37], and *Serratia Marcescens* [38]. Gram positive bacteria
293 are known as professional one in the production of BLM. The spore-forming bacteria like *B.*
294 *subtilis, B. licheniformis* and *Lysinibacillus louembei* have been widely used to produce BLM
295 [39-41].

296 Biosurfactants are native of several multicellular phenomena such as swarming described in
297 several bacterial species [42-45]. By using specific culture media we have shown that all strain
298 of *Shigella* genus were positive in swarming assay. The swarming phenomenon promote the
299 ability for biosurfactants production. Since this phenomenon is associated with either antibiotic
300 resistance, virulence and biofilm formation in *Proteus mirabilis*, *Salmonella enterica* serovar
301 Typhimurium and *Serratia* [46-48]. This idea reinforces the fact that *Shigella* sp. could also use
302 biosurfactants in its pathogenicity. No genes have been identified to be directly involved in
303 BLM biosynthesis. In this work we found that *ygaG* is a chromosomal gene of *S. flexneri* M90T.
304 YgaG which is the product of this gene shares 90 % of identity with LuxS involving in quorum
305 sensing and biofilm formation [49, 50]. RhlA, RhlB and RhlR proteins are known to promote

306 the rhamnolipid secretion [51]. The secretion of biosurfactant are correlated with quorum
307 sensing [52].

308 Pathogenicity in genus *Shigella* is determined by T3SS that has the ability to secrete myriad of
309 effector proteins into the target cell [11, 53]. In the absence of cellular contact, the secretory
310 apparatus is not functional [54], however some proteins are secreted in leak condition. Cell
311 contact is mimicking by the use of Congo red [55]. Under the Congo red induction condition,
312 all *Shigella* strains emulsified gasoline and diesel fuels while the *S. flexneri* 5a M90T spa40
313 mutant did not emulsify anymore. The mutant *S. flexneri spa40*- has not T3SS [26]. The *S.*
314 *flexneri spa40*- in non-inducible condition [56] or in a Congo red induction condition, does not
315 produce BLM. In addition by blocking T3SS using benzoic and salycilic acids compounds, we
316 have demonstrated that BLM could not be secreted in extracellular medium. This is confirm
317 that BLM is secreted via T3SS. *P. aeruginosa* could secrete in the presence or in the absence
318 of inhibitors. This allows us to postulate that rhamnolipid molecule could use another pathway.
319 An efflux mechanism is the top in *P. aeruginosa* [25, 57]. This inhibition assay with benzoic
320 acid and Salycilic acids showed a perfect correlation between the secretion of the BLM
321 synthesized by *Shigella* and the inactivation of the type III secretion apparatus.

322 Regarding the BLM characteristics, precipitation assay like hydrochloric acid, ammonium
323 sulfate and ethanol allowed to postulate that the secreted BLM could have a lipopeptide or
324 peptide features. Only peptide or lipopeptide biosurfactants can precipitate at very low pH or
325 with ammonium sulphate [22, 58]. In proteomics studies, the sequential precipitation of
326 ammonium sulfate proteins allows the proteins to be separated by "salting-in" or "salting-out"
327 effect [59], which necessarily leads to the formation of protein aggregates and therefore to their
328 precipitation. The BLM precipitate was able to emulsify gasoline and diesel fuel. Biosurfactants
329 like rhamnolipid, surfactin, emulsan are extractable by organic solvents [35, 60]. In addition

our study showed that the biosurfactant excreted by *Shigella sp.* is extractable with chloroform with higher efficiency and stability at 40 ° C.

BML are known to play several vital roles especially in microbe's adhesion, bioavailability, desorption and defense strategy. The most important role of microbial BLM is well reviewed for adhesion of the interfaces cells-cells interactions [61]. *P. aeruginosa* is a best example of cell surface hydrophobicity that justifies by the presence of cell-bound rhamnolipid [62]. Our new finding showed that by secreting BLM, *Shigella* sp. can easily bind to the cell hydrophobic interfaces by interacting with lipid rafts [27, 63-65]. By binding on cell membrane, BLM allows to reduce the membrane tension and to help the translocon like IpaB-C [66, 67] and the tip component IpaD [29, 32] to be closed to the host membrane and automatically to be inserted inside cytoplasmic membrane.

Many mechanisms have been demonstrated how *S. flexneri* can disseminate inside epithelial cells [68, 69], helping to escape autophagy phenomenon [70] and to spread inside host cell [71] by using a specific domain of IcsB that interacts with cholesterol [27]. In this work we showed that *S. flexneri*, *S. boydii* and *S. sonnei* can spread using swarming phenomenon. No studies have been previously documented about this strategy. This is efficiently emphasized and amplified the idea that *Shigella* is able to use several mechanisms that help *Shigella* to spread from cell to cell by secreting BLM. We are investigating the secretion of BLM inside epithelial cells. Basing on our finding, we can make a proposal that *Shigella* can invade and disseminate inside epithelial cells by using BLM.

Conclusion

In order to contribute to the understanding of the mechanism of invasion of epithelial cells by *Shigella sp.* we have first shown that all *Shigella* strain as well as clinical strain or

environmental strain are able to secrete biosurfactant like molecules directly in the extracellular. Secondly, we have shown that the secretion of biosurfactants like molecule are depending on Type Three Secretion System (T3SS). Our study suggest that, the biosurfactant with lipopetide or peptide features, stable at 40°C, could play an outstanding role on *Shigella* pathogenicity mechanisms including bacteria-host cell interaction, cell metabolism and cell dissemination. This work open ways to the understanding of genes associated with a couple of component that are able to promote the biosynthesis, the regulation and the secretion of BLM. MALDI-TOF and HPLC will be interesting to be done in order to characterize BLM.

Data Availability

The Excel and Prism7 sheet including the data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any intellectual commercial or financial relationships that could be construed as potential conflicts of interest.

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References

1. Organization WH. Food Safety: issues-public health significance of foodborne illnesses. Food Safety Programme. Regional Office for the Western Pacific. 2003.
2. Karen L Kotloff MSR, James A Platts-Mills, Patricia Pavlinac, Anita K M Zaidi. Shigellosis. Lancet 2018;391: 801–12

3. Collaborators GDD. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study Lancet Infect Dis 2017. 2015;17:909–48.
4. Kotloff KL NJ, Blackwelder WC, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 2013;382:209-22.
5. Liu J P-MJ, Juma J, et al. . Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. Lancet. 2016;388:1291-301.
6. Kotloff KL WJ, Ivanoff B, et al. . Global burden of shigella infections: implications for vaccine development and implementation. Bull World Health Organ. 1999;77:651-56.
7. Benoit S. Marteyn ADGaPJS. Shigella A model of virulence regulation in vivo. Gut Microbes 2012;3(2): 104–20. doi: http://dx.doi.org/10.4161/gmic.19325.
8. DuPont HL LM, Hornick RB, Formal SB. Inoculum size in shigellosis and implications for expected mode of transmission. J Infect Dis. 1989;159:1126-28.
9. Sansonetti PJ, Arondel, J., Cavaillon, J. M., & Huerre, M. . Role of interleukin-1 in the pathogenesis of experimental shigellosis. J Clin Invest. 1995;96:884-92.
10. Hale TL. Genetic basis of virulence in Shigella species. Microbiol Rev. 1998;55:206-24.
11. Kayath AC, Hussey, S., hajjami, N. E., Nagra, K., Philpott, D., & Allaoui, A. . Escape of intracellular Shigella from autophagy requires binding to cholesterol through the type III effector, IcsB. Microbes and Infection. 2010;12:956 - 66.
12. Parsot C, Menard, R., Gounon, P., & Sansonetti, P. J. . Enhanced secretion through the Shigella flexneri Mxi-Spa translocon leads to assembly of extracellular proteins into macromolecular structures. Mol Microbiol. 1995;16:291-300.
13. Kayath CA, Ibala Zamba A, Goma-Tchimbakala J, Mamonekene V, Mombo Makanga GM, Lebonguy AA, et al. Microbiota Landscape of Gut System of Guppy Fish (Poecilia reticulata) Plays an Outstanding Role in Adaptation Mechanisms. Int J Microbiol. 2019;2019:3590584. Epub 2019/04/06. doi: 10.1155/2019/3590584. PubMed PMID: 30949206; PubMed Central PMCID: PMCPMC6425301.
14. Roychoudhury SYaK. Emulsifying and flocculating performances of exopolysacharides produced by *Bacillus subtilis* strains and *Shigella flexneri*. International Journal of Research and Analytical Reviews (IJRAR) 2018;5(4):453-6.
15. Marchant RB, I.M. . Microbial biosurfactants: Challenges and opportunities for future exploitation. Trends Biotechnol. 2012;30:558-65.
16. Banat IMM, R.S.; Cameotra, S.S. . Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol. 2000;53:495-508.
17. Cameotra S, & Makkar, R. . Recent applications of biosurfactants as biological and immunological molecules. Current Opinion in Microbiology. 2004;7(3):262-6.
18. Déziel E, Lépine, F., Milot, S., & Villemur., R. rhlA is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology. 2003;149:2005–13.
19. Makaya PN, Guessennd, N. K., Kayath, C. A., Gba, K. K., Gbonon, V., Nguetta, S.-P. A., & Dosso, M. Emergence of Antibiotic Resistance and Correlation with the Efflux Pump in Pseudomonas aeruginosa Isolated from Abidjan Hospital. International Journal of Science and Research (IJSR). 2017;6(3):481-90.
20. Sharma M, & Arnaud, S. K. Swarming: A coordinnated bacterial activity. Curr Sci. 2002;83:707-15.
21. Rosenberg M, Gutnick, D., & Rosenberg, E. . Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrphobicity. FEMS Microbiology Letters. 1980;9:29-33.
22. Vater J, Kablitz, B., Wilde, C., Franke, P., Mehta, N., & Cameotra, S. . Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of Bacillus subtilis C-1 isolated from petroleum sludge. Appl Environ Microbiol. 2002;68:6210–9.

431 23. Fischer JR, Paukert CP. Habitat relationships with fish assemblages in minimally disturbed
432 Great Plains regions. Ecology of Freshwater Fish. 2008;17(4):597–609.
433 24. Ter Braak CJF, Šmilauer P. Canoco 4. Cambridge University Press, New York, NY, USA. 2003.
434 25. Usman MM, Dadrasnia, A., Lim, K. T., Mahmud, A. F., & Ismail, S. Application of
435 biosurfactants in environmental biotechnology, remediation of oil and heavy metal. AIMS
436 Bioengineering. 3(3):289-304.
437 26. Botteaux A, Kayath CA, Page AL, Jouihri N, Sani M, Boekema E, et al. The 33 carboxyl-terminal
438 residues of Spa40 orchestrate the multi-step assembly process of the type III secretion needle
439 complex in Shigella flexneri. Microbiology. 2010;156(Pt 9):2807-17. Epub 2010/05/29. doi:
440 10.1099/mic.0.039651-0. PubMed PMID: 20507885.
441 27. Kayath CA, Hussey S, El hajjami N, Nagra K, Philpott D, Allaoui A. Escape of intracellular
442 Shigella from autophagy requires binding to cholesterol through the type III effector, IcsB. Microbes
443 Infect. 2010;12(12-13):956-66. Epub 2010/07/06. doi: 10.1016/j.micinf.2010.06.006. PubMed PMID:
444 20599519.
445 28. Botteaux A, Sani M, Kayath CA, Boekema EJ, Allaoui A. Spa32 interaction with the inner-
446 membrane Spa40 component of the type III secretion system of Shigella flexneri is required for the
447 control of the needle length by a molecular tape measure mechanism. Mol Microbiol.
448 2008;70(6):1515-28. Epub 2008/11/21. doi: 10.1111/j.1365-2958.2008.06499.x. PubMed PMID:
449 19019157.
450 29. Schiavolin L, Meghraoui A, Cherradi Y, Biskri L, Botteaux A, Allaoui A. Functional insights into
451 the Shigella type III needle tip IpaD in secretion control and cell contact. Mol Microbiol.
452 2013;88(2):268-82. Epub 2013/02/21. doi: 10.1111/mmi.12185. PubMed PMID: 23421804.
453 30. El Hajjami N, Moussa S, Houssa J, Monteyne D, Perez-Morga D, Botteaux A. The inner-rod
454 component of Shigella flexneri type 3 secretion system, MxiI, is involved in the transmission of the
455 secretion activation signal by its interaction with MxiC. Microbiologyopen. 2018;7(1). Epub
456 2017/12/02. doi: 10.1002/mbo3.520. PubMed PMID: 29194994; PubMed Central PMCID:
457 PMCPMC5822323.
458 31. Cherradi Y, Schiavolin L, Moussa S, Meghraoui A, Meksem A, Biskri L, et al. Interplay between
459 predicted inner-rod and gatekeeper in controlling substrate specificity of the type III secretion
460 system. Mol Microbiol. 2013;87(6):1183-99. Epub 2013/01/23. doi: 10.1111/mmi.12158. PubMed
461 PMID: 23336839.
462 32. Sani M, Botteaux A, Parsot C, Sansonetti P, Boekema EJ, Allaoui A. IpaD is localized at the tip
463 of the Shigella flexneri type III secretion apparatus. Biochim Biophys Acta. 2007;1770(2):307-11. Epub
464 2006/11/18. doi: 10.1016/j.bbagen.2006.10.007. PubMed PMID: 17110044.
465 33. Botteaux A, Sory MP, Biskri L, Parsot C, Allaoui A. MxiC is secreted by and controls the
466 substrate specificity of the Shigella flexneri type III secretion apparatus. Mol Microbiol.
467 2009;71(2):449-60. Epub 2008/11/20. doi: 10.1111/j.1365-2958.2008.06537.x. PubMed PMID:
468 19017268.
469 34. Zdarta A, Smulek W, Trzcinska A, Cybulski Z, Kaczorek E. Properties and potential application
470 of efficient biosurfactant produced by Pseudomonas sp. KZ1 strain. J Environ Sci Health A Tox Hazard
471 Subst Environ Eng. 2019;54(2):110-7. Epub 2019/01/08. doi: 10.1080/10934529.2018.1530537.
472 PubMed PMID: 30614383.
473 35. Varjani SJ, Upasani VN. Critical review on biosurfactant analysis, purification and
474 characterization using rhamnolipid as a model biosurfactant. Bioresour Technol. 2017;232:389-97.
475 Epub 2017/02/28. doi: 10.1016/j.biortech.2017.02.047. PubMed PMID: 28238638.
476 36. Rossi EM, Beilke L, Kochhann M, Sarzi DH, Tondo EC. Biosurfactant Produced by Salmonella
477 Enteritidis SE86 Can Increase Adherence and Resistance to Sanitizers on Lettuce Leaves (Lactuca
478 sativa L., cichoraceae). Front Microbiol. 2016;7:9. Epub 2016/02/03. doi: 10.3389/fmicb.2016.00009.
479 PubMed PMID: 26834727; PubMed Central PMCID: PMCPMC4722381.
480 37. Chen G, Qiao M, Zhang H, Zhu H. Bacterial desorption in water-saturated porous media in the
481 presence of rhamnolipid biosurfactant. Res Microbiol. 2004;155(8):655-61. Epub 2004/09/24. doi:
482 10.1016/j.resmic.2004.05.001. PubMed PMID: 15380553.

38. Araujo HWC, Andrade RFS, Montero-Rodriguez D, Rubio-Ribeaux D, Alves da Silva CA, Campos-Takaki GM. Sustainable biosurfactant produced by Serratia marcescens UCP 1549 and its suitability for agricultural and marine bioremediation applications. Microb Cell Fact. 2019;18(1):2. Epub 2019/01/06. doi: 10.1186/s12934-018-1046-0. PubMed PMID: 30609918; PubMed Central PMCID: PMCPMC6318876.
39. Cooper DG, & Goldenberg, B. G. Surfaceactive agents from two Bacillus species. Appl Environ Microbiol. 1987;53:224-9.
40. Satpute SK, Banpurkar, A. G., Dhakephalkar, P. K., Banat, I. M., & Chopade, B. A. . Methods for investigating biosurfactants and bioemulsifiers: a review. Critical Reviews in Biotechnology. 2010;30(2):127–44.
41. Kaya-Ongoto MD, Kayath CA, Mbozo ABV, Mitoko GM, Wilson SPE, Kinavouidi DJK, et al. Prime Enzymatic Exocellular Background of Lysinibacillus louembei . . Advances in Microbiology , . 2020;95-109(10).
42. Déziel E, Lépine, F., Milot, S., & Villemur., R. rhlA is required for the production of a novel biosurfactant promoting swarming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology,. 2003;149:2005–13.
43. Jarrell KF, & McBride, M. J. The surprisingly diverse ways that prokaryotes move. Nat Rev Microbiol. 2008;(6):466–76.
44. Verstraeten N. Living on a surface: swarming and biofilm formation. Trends Microbiol. 16:496–506.
45. Kearns DB. A field guide to bacterial swarming motility. Nat Rev Microbiol. 8:634–44.
46. Belas R, & Suvanasuthi., R. . The ability of Proteus mirabilis to sense surfaces and regulate virulence gene expression involves FliL, a flagellar basal body protein. J Bacteriol. 2005;187:6789–803.
47. Williamson NR, Fineran, P. C., Ogawa, W., Woodley, L. R., & Salmond, G. P. . Integrated regulation involving quorum sensing, a two-component system, a GGDEF/EAL domain protein and a post-transcriptional regulator controls swarming and RhlA-dependent surfactant biosynthesis in Serratia. Environ Microbiol. 2008;10:1202–17.
48. Butler MT, Wang, Q., & Harshey, R. M. . Cell density and mobility protect swarming bacteria against antibiotics. Proc Natl Acad Sci U S A. 2010;107:3776–81.
49. Day WA, Jr., Maurelli AT. Shigella flexneri LuxS quorum-sensing system modulates virB expression but is not essential for virulence. Infect Immun. 2001;69(1):15-23. Epub 2000/12/19. doi: 10.1128/IAI.69.1.15-23.2001. PubMed PMID: 11119484; PubMed Central PMCID: PMCPMC97850.
50. Brito PH, Rocha EP, Xavier KB, Gordo I. Natural genome diversity of AI-2 quorum sensing in Escherichia coli: conserved signal production but labile signal reception. Genome Biol Evol. 2013;5(1):16-30. Epub 2012/12/19. doi: 10.1093/gbe/evs122. PubMed PMID: 23246794; PubMed Central PMCID: PMCPMC3595036.
51. Soberon-Chavez G, Aguirre-Ramirez M, Sanchez R. The Pseudomonas aeruginosa RhlA enzyme is involved in rhamnolipid and polyhydroxyalkanoate production. J Ind Microbiol Biotechnol. 2005;32(11-12):675-7. Epub 2005/06/07. doi: 10.1007/s10295-005-0243-0. PubMed PMID: 15937697.
52. Pearson JP, Pesci EC, Iglewski BH. Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J Bacteriol. 1997;179(18):5756-67. Epub 1997/09/19. doi: 10.1128/jb.179.18.5756-5767.1997. PubMed PMID: 9294432; PubMed Central PMCID: PMCPMC179464.
53. Sansonetti PJ, Arondel, J., Cantey, J. R., Prevost, M. C., & Huerre, M. . Infection of rabbit Peyer's patches by Shigella flexneri: effect of adhesive or invasive bacterial phenotypes on follicle-associated epithelium. Infect Immun. 1996;64:2752-64.
54. Ahalieyah Anantharajah M-PM-L. Targeting theTypeThree Secretion System in Pseudomonas aeruginosa. Trends in Pharmacological Sciences Cell Press. 2016.
55. Botteaux A, Sani, M., Kayath, C. A., Boekema, E. J., & Allaoui, A. . Spa32 interaction with the inner-membrane Spa40 component of the type III secretion system of Shigella flexneri is required for

536 the control of the needle length by a molecular tape measure mechanism. Molecular Microbiology.
537 2008;70(6):1515–28.
538 56. Parsot C. Shigella type III secretion effectors: how, where, when, for what purposes? . Curr
539 Opin Microbiol. 2009;12:110-6.
540 57. Ochsner UA, & Reiser, J. . Autoinducer-mediated regulation of rhamnolipid biosurfactant
541 synthesis in Pseudomonas aeruginosa. Proc Natl Acad Sci USA. 1995;92(14):6424-8.
542 58. Banat IM, Smyth, T. J., Perfumo, A., McClean, S., & Marchant, R. . Isolation and Analysis of
543 Lipopeptides and High Molecular Weight Biosurfactants. 2010.
544 59. Green A. Protein solubility on the basis of solubility in aqueous solutions of salts and organic
545 solvents. Methods Enzymol 1995;1:67-90.
546 60. Singh P, Patil Y, Rale V. Biosurfactant production: emerging trends and promising strategies. J
547 Appl Microbiol. 2019;126(1):2-13. Epub 2018/08/02. doi: 10.1111/jam.14057. PubMed PMID:
548 30066414.
549 61. Cameotra SS, Makkar RS, Kaur J, Mehta SK. Synthesis of biosurfactants and their advantages
550 to microorganisms and mankind. Adv Exp Med Biol. 2010;672:261-80. Epub 2010/06/16. doi:
551 10.1007/978-1-4419-5979-9_20. PubMed PMID: 20545289.
552 62. Zhang Y, Miller RM. Effect of a Pseudomonas rhamnolipid biosurfactant on cell
553 hydrophobicity and biodegradation of octadecane. Appl Environ Microbiol. 1994;60(6):2101-6. Epub
554 1994/06/01. PubMed PMID: 8031099; PubMed Central PMCID: PMCPMC201607.
555 63. Lafont F, van der Goot FG. Bacterial invasion via lipid rafts. Cell Microbiol. 2005;7(5):613-20.
556 Epub 2005/04/21. doi: 10.1111/j.1462-5822.2005.00515.x. PubMed PMID: 15839890.
557 64. Edidin M. The state of lipid rafts: from model membranes to cells. Annu Rev Biophys Biomol
558 Struct. 2003;32:257-83. Epub 2003/01/25. doi: 10.1146/annurev.biophys.32.110601.142439.
559 PubMed PMID: 12543707.
560 65. Hayward RD, Cain RJ, McGhie EJ, Phillips N, Garner MJ, Koronakis V. Cholesterol binding by
561 the bacterial type III translocon is essential for virulence effector delivery into mammalian cells. Mol
562 Microbiol. 2005;56(3):590-603. Epub 2005/04/12. doi: 10.1111/j.1365-2958.2005.04568.x. PubMed
563 PMID: 15819617.
564 66. Yang SC, Hung CF, Aljuffali IA, Fang JY. The roles of the virulence factor IpaB in Shigella spp. in
565 the escape from immune cells and invasion of epithelial cells. Microbiol Res. 2015;181:43-51. Epub
566 2015/12/08. doi: 10.1016/j.micres.2015.08.006. PubMed PMID: 26640051.
567 67. Blocker A, Gounon P, Larquet E, Niebuhr K, Cabiaux V, Parsot C, et al. The tripartite type III
568 secreton of Shigella flexneri inserts IpaB and IpaC into host membranes. J Cell Biol. 1999;147(3):683-
569 93. Epub 1999/11/05. doi: 10.1083/jcb.147.3.683. PubMed PMID: 10545510; PubMed Central
570 PMCID: PMCPMC2151192.
571 68. Agaisse H. Molecular and Cellular Mechanisms of Shigella flexneri Dissemination. Front Cell
572 Infect Microbiol. 2016;6:29. Epub 2016/03/26. doi: 10.3389/fcimb.2016.00029. PubMed PMID:
573 27014639; PubMed Central PMCID: PMCPMC4786538.
574 69. Koestler BJ, Fisher CR, Payne SM. Formate Promotes Shigella Intercellular Spread and
575 Virulence Gene Expression. mBio. 2018;9(5). Epub 2018/09/27. doi: 10.1128/mBio.01777-18.
576 PubMed PMID: 30254126; PubMed Central PMCID: PMCPMC6156198.
577 70. Ogawa M, Suzuki T, Tatsuno I, Abe H, Sasakawa C. IcsB, secreted via the type III secretion
578 system, is chaperoned by IpgA and required at the post-invasion stage of Shigella pathogenicity. Mol
579 Microbiol. 2003;48(4):913-31. Epub 2003/05/20. doi: 10.1046/j.1365-2958.2003.03489.x. PubMed
580 PMID: 12753186.
581 71. Allaoui A, Mounier J, Prevost MC, Sansonetti PJ, Parsot C. icsB: a Shigella flexneri virulence
582 gene necessary for the lysis of protrusions during intercellular spread. Mol Microbiol.
583 1992;6(12):1605-16. Epub 1992/06/01. doi: 10.1111/j.1365-2958.1992.tb00885.x. PubMed PMID:
584 1495389.

585