Research Note

Growth of Enterotoxigenic *Bacillus cereus* on Salmon (*Oncorhynchus nerka*)

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ABSTRACT

We previously demonstrated the widespread presence of enterotoxigenic *Bacillus cereus* in marine foods. In view of the widespread consumption of raw fish, we sought to determine the ability of this organism to grow on the surface of wild Alaskan salmon at abusive temperatures (12, 16, and 20°C), using an isolate able to produce elevated levels of hemolysin BL enterotoxin and nonhemolytic enterotoxin. An incubation temperature of 37°C for colony formation was found to be selective for *B. cereus* grown on salmon held for up to 24 h at each temperature. A fivefold increase in log CFU per gram was observed after 26 and 22 h at 16 and 20°C, respectively, while a >4-log CFU/g increase occurred on salmon held at 12°C for 48 h. Generation times of 169.7, 53.5, and 45.6 min were observed at 12, 16, and 20°C. Nonhemolytic enterotoxin was detected when levels of *B. cereus* were in excess of 10⁶ CFU/g. Nisin, at concentrations of 1 and 15 μg/g of salmon, reduced levels of *B. cereus* 2.5- and 25-fold, respectively. Our results indicate that fresh salmon can serve as an excellent substrate for enterotoxigenic *B. cereus* and that this organism can reach levels associated with foodborne illness following moderate temperature abuse.

*Bacillus cereus* is a ubiquitous spore-forming, facultatively anaerobic bacterium that has been implicated in foodborne illness worldwide. The emetic syndrome is caused by cereulide, a 45-kDa cyclic heat-stable dodecapeptide, whereas in diarrheal cases at least two toxins, i.e., the three-component heat-labile enterotoxins hemolysin BL (HBL) and nonhemolytic enterotoxin (NHE), are produced by food poisoning strains (12) that are mostly mesophilic (13).

In the United States, approximately 15% of cases of foodborne illness involve seafood as the vehicle of transmission (21). Between 1990 and 2003, seafood was the most commonly identified vehicle of foodborne disease in the United States (30). In spite of public health advisories regarding the presence of microbial pathogens in raw protein foods (3, 22, 26), consumption of raw fish is an increasingly popular food group in western countries, whether consumed alone, such as sashimi, or in combination with other ingredients such as rice in sushi. We previously reported the widespread presence of enterotoxigenic *B. cereus* in retail rice, with 94% of isolates producing HBL or NHE (2). Indeed rice and other grains are a common source of enterotoxigenic *B. cereus* (18, 19, 29).

There have been at least two reported cases of foodborne illness due to *B. cereus* with salmon as the vehicle (6). We recently reported on the incidence of *B. cereus* in retail seafood (27). The organism was present at levels between 3.6 and >1,100 CFU/g in approximately 18% of samples. All but one isolate were of the diarrheal toxin type, and approximately one-half of the isolates detected produced both HBL and NHE. No isolate was able to grow at 7°C. In another survey, enterotoxigenic *B. cereus* was found in 29% of tropical fish and shellfish (8). Domenech-Sanchez et al. (10) recently described an outbreak of foodborne illness due to *B. cereus* after the consumption of temperature-abused tuna. To our knowledge there have been no previous studies assessing the ability of *B. cereus* to grow on marine foods, the objective of the present work. Here we demonstrate the ability of enterotoxigenic *B. cereus* to grow on one popular marine food, salmon, which is often consumed raw. Quantifying the risks associated with consumption of raw foods was a recently identified recommended area of consumer-related research in microbial food safety (1).

MATERIALS AND METHODS

Strains. *B. cereus* strain no. 23, isolated from “fish cracker” as described earlier (27), was used. Neither this isolate nor dozens of others we previously isolated from marine foods were able to grow at 7°C. This isolate was selected for this study based on its ability to produce both HBL and NHE at elevated levels and grow at 12°C. The inoculum was prepared by overnight growth in nutrient broth followed by inoculation into a flask containing 25 ml of nutrient broth with shaking at 32°C. Cells were collected at an *A*₆₀₀ of 0.60, corresponding to the mid-logarithmic phase of growth. To prepare the inoculum, cells (1.0 ml) were washed and resuspended in 1.0 ml of 0.01 M sodium phosphate buffer, pH 7.0, and then diluted 1:1,000 in the same buffer.

Substrate. Packaged, frozen fillets of wild Alaskan red salmon (*Oncorhynchus nerka*) were obtained from a local
supermarket and held frozen at $-20^\circ$C until used. Thin sections of the top and bottom surfaces of the partially thawed salmon were aseptically removed, after which, with a sterile cork borer, 2.54-cm (1-in.)-diameter sections (cores) were removed, corresponding to approximately 3.5 g.

**Identification of indigenous microflora.** Using phosphate buffer, serial dilutions of thawed, whole salmon were prepared, and the members of the indigenous microflora were enumerated on nutrient agar. The two predominant isolates were identified by a commercial laboratory by 16S RNA sequencing (Accugenix, Newark, DE).

**Growth on fish.** Fifty microliters of diluted *B. cereus* inoculum was evenly added to the surface of cored salmon pieces, previously tempered to the appropriate temperature, at an initial level of between $1.0 \times 10^3$ to $2.0 \times 10^3$ CFU/g of salmon. These levels correspond to levels we previously observed in retail seafood (27). Samples (3.5 g) were held at 12, 16, and 20°C, representing varying levels of temperature abuse. At appropriate times samples were macerated in 0.1% peptone in a stomacher, diluted in 0.1% peptone, and pour plated in duplicate with nutrient agar. The two predominant isolates were identified by a library requiring the use of a selective medium to inhibit background flora on plates held at 37°C, were obtained for salmon held at 16 and 20°C for 24 h before analysis (not shown). The absence of background microflora on plates held at 37°C indicates that this temperature may be a convenient and economical selective tool for challenge studies of mesophilic *B. cereus* for up to approximately 24-h observation times, at least in the case of certain marine foods. However, cored samples of salmon held for 48 h at 12°C (Table 1) before analysis produced $10^3$ to $10^5$ CFU/g on plates incubated at 20 or 32°C. However, such background microorganisms did not appear if plates were incubated at 37°C up to 48 h (Table 1). Similar results, i.e., the absence of background flora on plates held at 37°C, were obtained for salmon held at 16 and 20°C for 24 h before analysis (not shown). The absence of background microflora on plates incubated at 37°C indicates that this temperature may be a convenient and economical selective tool for challenge studies of mesophilic *B. cereus* for up to approximately 24-h observation times, at least in the case of certain marine foods. However, cored samples of salmon held for 48 h at either 12°C (Table 1) or 16°C (not shown) before analysis contained $>10^4$ CFU/g even on plates incubated at 37°C, requiring the use of a selective medium to inhibit background flora. We (20) and others (11) have found the chromogenic agar used here to be more effective in suppressing high levels of background microflora than the older *B. cereus* selective medium (mannitol egg yolk polymyxin agar) (31). The high cost of the chromogenic medium is a further incentive for using 37°C as a selective temperature when possible for mesophilic *B. cereus*.

**Detection of *B. cereus*.** We next determined the ability of *B. cereus* to grow on cored sections of salmon held at 12,

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**RESULTS AND DISCUSSION**

**Characteristics of salmon and background microflora.** The directly measured pH of macerated, cored salmon was 6.3 ± 0.05, and the $a_w$ was 0.991 ± 0.003. The lower limits of pH and $a_w$ for growth of *B. cereus* are reported to be between 4.35 and 4.90 and between 0.912 and 0.950, respectively (19). We next identified the predominant microflora organisms associated with whole, freshly thawed salmon fillets. A value of $1.3 \times 10^7 \pm 0.1 \times 10^5$ CFU/g was obtained with nutrient agar plates held at 20°C for 48 h. Two predominant, pigmented, rod-shaped isolates were observed; one was identified as *Chrysobacterium* (formerly *Flavobacterium*) piscium, and the other is an unidentified species in the order *Flavobacteria*. Environmental flavobacterial isolates are typically isolated from marine and freshwater environments (15, 17).

Aerobic plate counts of freshly prepared, cored salmon samples contained numbers below the detection limit ($10^2$/g) at zero time even on plates held for 48 h to detect slow-growing members of the microflora (not shown). Cored samples of salmon each held for 24 h at 12°C (Table 1) before analysis produced $10^3$ to $10^5$ CFU/g on plates incubated at 20 or 32°C. However, such background microorganisms did not appear if plates were incubated at 37°C up to 48 h (Table 1). Significant results, i.e., the absence of background flora on plates held at 37°C, were obtained for salmon held at 16 and 20°C for 24 h before analysis (not shown). The absence of background microflora on plates incubated at 37°C indicates that this temperature may be a convenient and economical selective tool for challenge studies of mesophilic *B. cereus* for up to approximately 24-h observation times, at least in the case of certain marine foods. However, cored samples of salmon held for 48 h at either 12°C (Table 1) or 16°C (not shown) before analysis contained $>10^4$ CFU/g even on plates incubated at 37°C, requiring the use of a selective medium to inhibit background flora. We (20) and others (11) have found the chromogenic agar used here to be more effective in suppressing high levels of background microflora than the older *B. cereus* selective medium (mannitol egg yolk polymyxin agar) (31). The high cost of the chromogenic medium is a further incentive for using 37°C as a selective temperature when possible for mesophilic *B. cereus*.

**Detection of *B. cereus*.** We next determined the ability of *B. cereus* to grow on cored sections of salmon held at 12,
16, and 20°C, separately. Because of its selective ability, an incubation temperature of 37°C for plates was used in the work described below, which assessed the ability of *B. cereus* to grow on salmon at 16 and 20°C. Initial inoculum levels corresponded to levels of *B. cereus* previously reported for *B. cereus* on retail seafood (27). Lag times of 2 and 6 h were observed at holding temperatures of 20 and 16°C, respectively (Fig. 1). Because of the slow growth, no attempt was made to determine the lag phase at 12°C. No growth was observed on salmon held at 7°C (not shown). The maximum observed values in CFU per gram at 16 and 20°C were approximately $2.5 \times 10^8$ in each case at 26 and 22 h, respectively. Generation times at each temperature were obtained from the slope of the logarithmic phase of growth. These were 169.7, 53.5, and 45.6 min at 12, 16, and 20°C, respectively. A generation time of 45.6 min at 20°C indicates the ability of this foodborne pathogen to grow readily on salmon at moderate temperature abuse conditions, with levels of $>10^3$ CFU/g within 22 h at this temperature. At an incubation temperature of 12°C, 45 h was required to reach levels of $10^7$ to $10^8$ CFU/g. These levels of enterotoxigenic *B. cereus* are sufficient to cause foodborne illness.

**Enterotoxin production.** NHE on salmon was first detected after 20 h at 20°C and after 26 h at 16°C, corresponding to $1.2 \times 10^8$ and $2.0 \times 10^8$ CFU/g, respectively. The *B. cereus* strain used in this study was the highest NHE-producing isolate obtained from retail seafood isolates (27). The total number of ingested *B. cereus* cells required to produce the diarrheal illness is believed to be in the range of $10^5$ to $10^8$ (12). Preformed enterotoxin is not believed to be involved in foodborne illness caused by diarrheal strains of *B. cereus* but rather through the production of the enterotoxin in the intestine by ingested cells (12).

**Nisin.** At the concentrations used, nisin resulted in a significant ($P < 0.05$) inhibition of growth of *B. cereus* on salmon held at 16°C for 48 h. At a concentration of 1 μg/g, nisin resulted in an approximate 2.5-fold reduction in cell numbers of *B. cereus*, while an ~25-fold reduction was observed at a nisin concentration of 15 μg/g (Table 2). Nisin has been found to inhibit *B. cereus* in beef gravy (4) as well as *Listeria monocytogenes* on smoked salmon when used with other hurdles such as other antimicrobials (9, 24), vacuum packaging (23), or modified atmosphere (25).

Although fresh salmon has been identified as a vehicle in foodborne illness due to *Salmonella* (5), there has been little work on its ability to support the growth of other pathogens. Its potential as a substrate for microbial growth is not surprising given its nutrient content, moderate pH level, and high aw. The widespread presence of *B. cereus* in marine foods identifies it as a potential food poisoning agent from fresh marine species. The most common hurdle in the handling of raw marine foods intended for human consumption is low temperature. Surveys have repeatedly found that 10 to 20% of refrigerators and retail display cases exceed 10°C (7, 14, 16, 28, 32). This indicates that *B. cereus* (and other mesophilic foodborne pathogens) could grow to elevated levels on marine foods following moderate temperature abuse, as has been reported in a case in which tuna was responsible for *B. cereus* foodborne illness following temperature abuse (10). In the case of spore-forming, foodborne pathogens, an intrinsic safety factor in addition to low temperature may be spore germination time and ability, should such pathogens be present in the spore state and remain dormant due to the lack of the appropriate germinant. It will be of interest to determine the ability of enterotoxigenic *B. cereus* to grow on other marine foods commonly consumed raw.

**ACKNOWLEDGMENTS**

This study was supported by a U.S. Department of Agriculture Seafood Safety Grant. The assistance of Mark Normand with the creation of the graphics file is appreciated.

**REFERENCES**


