Ability of Shiga Toxin-Producing *Escherichia coli* and *Salmonella* spp. To Survive in a Desiccation Model System and in Dry Foods

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In order to determine desiccation tolerances of bacterial strains, the survival of 58 diarrheagenic strains (18 salmonellae, 35 Shiga toxin-producing *Escherichia coli* [STEC], and 5 shigellae) and of 15 nonpathogenic *E. coli* strains was determined after drying at 35°C for 24 h in paper disks. At an inoculum level of 10^7 CFU/disk, most of the salmonellae (14/18) and the STEC strains (31/35) survived with a population of 10^3 to 10^4 CFU/disk, whereas all of the shigellae (5/5) and the majority of the nonpathogenic *E. coli* strains (9/15) did not survive (the population was decreased to less than the detection limit of 10^2 CFU/disk). After 22 to 24 months of subsequent storage at 4°C, all of the selected salmonellae (4/4) and most of the selected STEC strains (12/15) survived, keeping the original populations (10^3 to 10^4 CFU/disk). In contrast to the case for storage at 4°C, all of 15 selected strains (5 strains each of *Salmonella* spp., STEC O157, and STEC O26) died after 35 to 70 days of storage at 25°C and 35°C. The survival rates of all of these 15 strains in paper disks after the 24 h of drying were substantially increased (10 to 79 times) by the presence of sucrose (12% to 36%). All of these 15 desiccated strains in paper disks survived after exposure to 70°C for 5 h. The populations of these 15 strains inoculated in dried foods containing sucrose and/or fat (e.g., chocolate) were 100 times higher than those in the dried paper disks after drying for 24 h at 25°C.

A nationwide outbreak of gastroenteritis due to consumption of dried squid chips (water activity, 0.5 to 0.6) contaminated with *Salmonella enterica* serovars Oranienburg and Chester occurred in Japan in 1999, in which a total of 1,634 cases were reported from all over the country (46/47 prefectures) (25). Even though this outbreak was the first one caused by contaminated dry foods in Japan, a number of outbreaks of gastroenteritis caused by dry foods such as dried milk, chocolate, potato chips, and almonds contaminated mainly by salmonellae have been reported in the United States and Europe since the 1960s (5, 12, 16, 18). Since the middle of the 1990s, outbreaks of Shiga toxin-producing *Escherichia coli* (STEC) O157 infections have also been reported to be associated with dry foods such as deer jerky and salami in the United States (8, 14, 19, 24). Many outbreaks of gastroenteritis associated with dry foods contaminated with salmonellae and STEC strains have been reported all over the world, even though the bacterial growth was inhibited by low water activity (a_w) in these dry foods. Accordingly, we tried to investigate the ability of these desiccated strains to survive under dried conditions, using salmonellae and STEC O157, O26, and O111 strains in paper disks with a_w s of 0.5 to 0.6 and in selected dry foods. In order to study the effect of bacterial environments on the survival of bacteria during a process of being made “desiccated” bacteria and of storage conditions on the survival of desiccated bacteria produced after 24 h of drying, we examined the effects of pH, food ingredients such as sucrose, storage period, and storage temperature, using a dried paper disk (with constant a_w levels between 0.5 and 0.6) model. We also studied the survival of

these desiccated bacteria in selected dry foods after artificial inoculation.

MATERIALS AND METHODS

Bacterial strains. A total of 58 diarrheagenic bacterial isolates (56 from patients with diarrhea collected between 1996 and 2001 and 2 from the dried squid chips incriminated in the diffuse outbreak of salmonellae in Japan) were utilized in this study (Table 1). Each of these 58 strains except the 2 strains from the squid chips (*Salmonella* serovars Oranienburg [strain 1] and Chester [strain 2]) was isolated from geographically and chronologically different case: 18 strains of *Salmonella* spp. were isolated from 16 sporadic cases and from one outbreak case in which *Salmonella* serovars Oranienburg and Chester were detected from the dried squid chips, 15 strains of STEC O157 were isolated from 13 sporadic and 2 outbreak cases, 15 strains of STEC O26 were from 15 sporadic cases, 5 strains of STEC O111 were from 5 sporadic cases, and 5 strains of *Shigella* spp. were from 5 sporadic cases. In order to compare the abilities of these strains to survive dry conditions, 15 isolates of nonpathogenic *E. coli* (Table 1) were also examined. These 15 nonpathogenic *E. coli* strains had been isolated in our laboratory from stools of 15 healthy persons collected in Aichi prefecture, Japan, between 1999 and 2001.

Dry foods utilized for the assessment of resistance of inoculated bacteria to dryness. Survival of bacteria inoculated and dried on dry foods was examined using seven different kinds of marketed dry foods, including chocolates, roasted peanuts, potato chips, and dried squid chips (seasoned with soy sauce and sucrose), all of which had been reported as being responsible for outbreaks of gastroenteritis previously (12, 13, 16, 23, 25), together with dried apples, plain sun-dried squids, and dried sour plum pickles (Japanese traditional pickles).

Preparation of desiccated bacteria in paper disks. Paper disks (10 mm in diameter) marketed in Japan (Advantec, Tokyo, Japan) for antibacterial susceptibility tests were autoclaved (121°C, 15 min) and then dried for 24 h in an incubator (HD-8-CP; Hirasawa Works, Tokyo, Japan) at 35°C. These paper disks (one to four disks placed in each sterile plastic plate [85 mm in diameter]) were seeded with 50 μ l of bacterial solutions prepared as follows: a fresh culture of each bacterial strain, which was preincubated overnight at 35°C on tryptic soy agar (Difco, Detroit, MI), was suspended in 10 ml of a sterile physiological saline (SPS), and then the bacterial population was adjusted by matching the density with a 0.5 to 1 McFarland standard (ca. 10^8 CFU/ml) (15). The seeded paper disks in the sterile plastic plates were dried for 24 h in an incubator at 35°C with plate covers completely closed following 5 min of slight opening to evaporate water. After the 24 h of drying, some of these plates were sealed with tape,

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TABLE 1. Bacterial strains tested

Group	Strain no.	Serovar or species	No. of strains
<i>S. enterica</i>	1	Oranienburg	1
	2	Chester	1
	3	Enteritidis	1
	4	Litchfield	1
	5	Anatum	1
	6	Infantis	1
	7	Istanbul	1
	8	Montevideo	1
	9	Nagoya	1
	10	Newport	1
	11	Saintpaul	1
	12	Senftenberg	1
	13	Typhimurium	1
	14	Virchow	1
	15–18	Typhi	4
STEC O157	19–27	H7 (Stx1,2)	9
	28–30	H7 (Stx2)	3
	31–33	H–(Stx2)	3
STEC O26	34–44	H11 (Stx1)	11
	45	H11 (Stx1,2)	1
	46,47	H–(Stx1)	2
	48	H–(Stx1,2)	1
STEC O111	49–52	H–(Stx1)	4
	53	H–(Stx1,2)	1
<i>Shigella</i> spp.	54	<i>S. flexneri</i> 2a	1
	55	<i>S. flexneri</i> 2b	1
	56	<i>S. flexneri</i> 3a	1
	57	<i>S. sonnei</i>	1
	58	<i>S. boydii</i> 3	1
Nonpathogenic <i>E. coli</i>	59–73		15

wrapped in plastic bags, and stored in a refrigerator (4°C) for up to 22 to 24 months.

Measurement of water activity and pH. The a_w of blank paper disks was measured using an a_w meter (WA-360; Shibaura Electronics Co., Ltd., Saitama, Japan) after drying at 35°C for 24 h following addition of 50 μ l of SPS instead of bacterial solutions. The a_w s of dried blank paper disks were also measured after 10, 30, and 70 days of storage at 4°C, 25°C, and 35°C and after 12 and 24 months of storage at 4°C. Water activities of the dry foods before and after drying at 25°C for 24 h following addition of 50 μ l of SPS were also determined. The pHs of the dry foods were determined with a pH meter (S881; Horiba Ltd., Kyoto, Japan) after homogenization of 10 g each of the dry foods with 10 ml of distilled water with a Stomacher 400 (Seward, London, United Kingdom).

Enumeration of desiccated bacteria surviving in paper disks. Each dried paper disk seeded with a selected strain of bacteria was placed in a sterile plastic bag containing 100 ml of SPS and then pummeled for 2 min using the Stomacher 400. Serial dilutions (1:10) of SPS were prepared, and 1 ml each of a serial dilution was transferred to a sterile plastic plate (85 mm in diameter) in duplicate and then mixed with 15 ml of autoclaved plate count agar (Difco, Detroit, MI) kept at 45°C. After solidification at room temperature, the plates were incubated for 48 h at 35°C in the incubator. The number of colonies was counted on duplicate plates in a suitable range (30 to 300 colonies/plate) according to the *Official Methods of Analysis of AOAC International* (1). If plates from all dilutions yielded fewer than 30 colonies on any plates, we counted the actual number of colonies generated on the plates seeded with the original bacterial solution without dilution. Populations of all of the 73 strains (58 pathogenic and 15 nonpathogenic strains) seeded in paper disks were determined after drying for 24 h at 35°C (Fig. 1, panel 1). After this initial drying, the dried paper disks with desiccated bacteria were stored in a refrigerator (4°C) for periods of 5 to 10 days, 20 to 40 days, 7 to 9 months, and 22 to 24 months, and then the bacterial populations of four selected strains of *Salmonella* spp.; five strains each of STEC O157, STEC O26, and STEC O111; and six strains of nonpathogenic *E. coli* in

the disks were determined at the end of each storage period (Fig. 1, panel 1). Our complete experimental protocols are shown in Fig. 1 as a flow chart.

Effect of environmental factors on desiccation tolerance of *Salmonella* spp. and STEC. In the following series of experiments, the preparations and enumerations of desiccated bacteria in paper disks were carried out by exactly the same methods as described above, using 15 selected strains (5 strains each of *Salmonella* spp. (strains 1 to 4 and 13), STEC O157 (strains 19 to 21, 28, and 31), and STEC O26 (strains 34 to 36, 46, and 47)).

(i) **Survival of the 15 strains after 24 h of drying at 35°C under different environmental conditions (Fig. 1, panel 2).** (a) **Effect of pH on survival.** One milliliter of 10 times concentrated bacterial solution (ca. 10^9 CFU/ml) containing one of each of these strains was added to 9 ml each of SPS with the pH adjusted with 1% acetic acid or 1 N NaOH. The pH values of the final bacterial solutions were adjusted to 3, 4, 5, 6, 7, 8, and 9. Populations of the 15 desiccated strains in the paper disks were determined after 24 h of drying following addition of 50 μ l each of the bacterial solution with the selected pH value.

(b) **Effect of sucrose and NaCl on survival.** One milliliter of concentrated bacterial solution (ca. 10^9 CFU/ml) was added to 9 ml each of SPS having three different sucrose concentrations. The sucrose concentrations of the final bacterial solutions were adjusted to 4, 12, and 36%. The population of a desiccated strain in each dried paper disk was determined exactly the same way as described above. The bacterial solution (1 ml) was added to an autoclaved cocoa drink (9 ml) containing the same sucrose concentrations (4, 12, and 36%) in order to study a possible difference between the effects of sucrose alone and sucrose plus lipids in the bacterial solution on the survival of these bacteria. The population of each strain in the paper disk was then also determined.

Effects of NaCl in the bacterial solutions (final concentrations of 5, 10, and 30%) were also determined by enumeration of strains, using the same enumeration method as described above.

(ii) **Survival of the 15 desiccated strains (Fig. 1, panel 3).** (a) **Effect of storage temperature on survival.** The populations of the 15 desiccated strains in the dried paper disks stored at 4°C, 25°C, and 35°C were determined after 1, 15, 35, and 70 days of storage.

(b) **Effect of ethanol on survival.** Fifty microliters each of 0, 5, 15, 40, and 70% (vol/vol in sterile distilled water) ethanol solutions was absorbed for 1 min onto a dried paper disk containing one each of the 15 desiccated bacteria. Each disk was then quickly placed in a sterile plastic bag containing 100 ml of SPS and then analyzed for the population of bacteria.

(c) **Effect of exposure to high temperature on survival.** A dried paper disk containing one each of the 15 desiccated strains in a sterilized glass plate (85 mm in diameter) was exposed to high temperatures of 70°C, 80°C, and 90°C for 5 h in a forced-air oven. After 5 h of heat exposure, the population of bacteria in each disk was determined compared with the corresponding control population, which was kept at 35°C for 5 h simultaneously.

Survival of *Salmonella* spp. and STEC in dry foods after 24 h of drying at 25°C. In this series of experiments, we used the same 15 selected strains as we used in the previous series of experiments. Approximately 1 g (0.8 to 1.2 g) of four different dry foods (chocolates, roasted peanuts, dried apples, and dried sour plum pickles) was inoculated with one each of the selected 15 bacterial solutions (50 μ l, ca. 10^8 CFU/ml) and then dried at 25°C for 24 h in a sterile plastic plate. Populations of the 15 strains in these dried foods and in dried disks for controls were determined by enumeration of the corresponding colonies. Four selected strains (two strains of *Salmonella* spp. isolated from the dried squid chips and one strain each of STEC O157 and O26) were investigated for their survival in three different dry foods (potato chips, dried squid chips, and plain sun dried squid), using the same procedure as described above (Fig. 1, panel 4).

RESULTS

Determination of water activities of dried disks and dry foods and measurement of pHs of dried foods. After 12 a_w determinations over an extended time course (0 day to 24 months) and different storage temperature (4°C, 25°C, and 35°C), we found that the a_w values of the disks were within a small range of variation of 0.56 ± 0.02 (mean $\pm$ standard deviation). The a_w values of seven different dry foods used in this study were found to be between 0.41 and 0.64. These values did not change significantly after drying at 25°C for 24 h following addition of 50 μ l of SPS, with the a_w values remaining between 0.43 and 0.58. With regard to pH, five out of the

1. Survivals after 24h drying on paper discs and subsequent storage for 24 months

2. Survivals after 24h drying on paper discs with different conditions

3. Survivals of bacteria after becoming "desiccated" in different conditions

4. Survivals of bacteria in dry foods after 24h drying

FIG. 1. Flow chart of the experimental procedures. #1, 10^7 CFU in sterile physiological saline; #2, 10^7 CFU in 5 to 30% NaCl; *, after storage for periods of 5 to 10 days, 20 to 40 days, 7 to 9 months, and 22 to 24 months; **, 10^7 CFU/g.

seven dry foods had pH values of between 5.75 and 6.56, except dried apples (pH 3.59) and pickled sour plums (pH 3.12).

Survival of bacteria in paper disks after 24 h of drying and survival of desiccated bacteria after refrigeration storage (4°C). The majority of the 18 *Salmonella* strains (14/18) inoc-

ulated at levels of 10^7 CFU/disk on paper disks survived at levels of 10^3 to 10^4 CFU/disk after the initial 24 h of drying at 35°C, except for *Salmonella enterica* serovar Typhi (4/4), which were extinguished to a level less than the detection limit (10^2 CFU/disk) after the drying (Fig. 2). Most of the STEC strains

FIG. 2. Survival of bacterial strains inoculated at 10^7 cells/disk on paper disks after 24 h of drying at 35°C . Each circle represents the number of each strain that survived. The bottom and top edges of the superimposed box plots are the 25th and 75th distribution percentiles, respectively; the central horizontal line is the median, and the central vertical lines show the range of the data. N-T, nontyphoid; N-P, non-pathogenic.

(31/35) also survived at levels of 10^3 to 10^4 CFU/disk after the 24 h of drying, regardless of the serotypes or toxin types (Fig. 2). One of the most noteworthy findings in this study was that after becoming desiccated through the 24 h of initial drying, 100% of selected salmonellae (4/4) and 80% of selected STEC strains (12/15) showed strong resistance to dryness in refrigerated storage conditions (4°C) (Table 2). In other words, all of the 4 *Salmonella* strains and all of the 15 STEC strains (5 each of STEC O157, O26, and O111), except 1 strain of STEC O26 and 2 strains of STEC O111, maintained almost the same population level as at the beginning of the refrigeration storage (10^3 to 10^4 CFU/disk) for up to 22 to 24 months (Table 2). In quite a contrast to a very high survival rate of STEC strains (89%), only 40% (6/15) of nonpathogenic *E. coli* strains survived the initial 24 h of drying (Fig. 2). In other words, the majority (60%) of nonpathogenic *E. coli* strains did not survive to become desiccated bacteria. Out of the six survivors of the 24 h of drying, however, five strains (two within 40 days and three within 9 months) were extinguished within 9 months of refrigerated storage (Table 2). Populations of all of the five *Shigella* strains decreased to a level less than the detection limit after the initial 24 h of drying (Fig. 2). In other words, none of the shigellae became desiccated.

Effect of environmental factors on survival of the selected 15 strains of *Salmonella* spp. and STEC (5 strains each of *Salmonella*, STEC O157, and O26). (i) **Survival of the 15 strains after 24 h of drying at 35°C with different environmental conditions.** (a) **Effect of pH on survival.** All of these 15 strains survived at levels of 10^3 to 10^4 CFU/disk within a pH range between 4 and 9 after 24 h of drying at 35°C following an inoculation of 10^7 CFU/disk. The populations of all of these 15 strains, however, decreased to below the detection level (10^2 CFU/disk) at pH 3 after the 24 h of dryness.

(b) **Effect of sucrose and NaCl on survival.** The populations (mean $\pm$ standard deviation) of the 15 strains in the paper disks after the 24 h of dryness following the addition of bac-

TABLE 2. Survival of desiccated bacteria after storage at 4°C in dried paper disks

Strain no.	Bacterial group	Log ₁₀ CFU/disk					
		Inoculated	Surviving	Surviving after storage for:			
				5–10 days	20–40 days	7–9 mo	22–24 mo
1	<i>Salmonella</i> spp.	6.95	4.52	4.32	4.66	4.40	4.52
2		7.00	3.49	3.88	3.40	3.15	2.95
3		6.93	3.96	4.08	4.28	4.11	4.08
4		6.93	4.43	4.53	4.48	4.77	3.98
19	STEC O157	6.93	4.04	4.26	4.32	4.28	4.36
20		6.85	4.51	4.45	4.45	4.26	4.18
21		7.78	5.40	4.93	5.08	4.34	3.85
28		6.90	4.68	4.04	3.99	4.08	4.00
31		7.57	5.23	5.23	5.00	4.32	3.94
34	STEC O26	7.57	5.26	4.67	4.72	4.28	4.36
35		7.48	4.15	3.04	3.94	3.18	<2.00
36		7.00	3.64	3.65	3.72	3.45	3.49
46		7.00	4.85	4.66	4.83	3.96	3.34
47		6.81	3.95	4.00	3.97	3.99	4.23
49	STEC O111	7.51	5.04	4.91	4.87	5.04	4.18
50		7.28	3.71	3.71	3.72	<2.00	<2.00
51		7.04	4.08	4.00	3.99	3.38	2.30
52		6.90	4.63	4.59	4.32	3.00	2.60
53		6.78	4.59	4.49	4.08	3.58	<2.00
59	Nonpathogenic <i>E. coli</i>	6.90	4.08	4.00	4.08	4.08	3.79
60		7.56	3.30	3.18	2.93	<2.00	NT ^a
61		7.18	3.87	3.54	3.08	<2.00	NT
62		7.00	3.60	3.34	2.70	<2.00	NT
63		7.15	3.63	3.54	<2.00	NT	NT
64		7.70	3.38	2.60	<2.00	NT	NT

^a NT, not tested.

terial solution with 4%, 12%, and 36% sucrose were 4.53 ± 0.20 , 5.30 ± 0.36 , and 5.59 ± 0.29 log₁₀ CFU/disk, respectively. These results indicated that survival of all of the *Salmonella* and STEC strains was substantially enhanced by the presence of sucrose (at 4% by 1.2 to 6.4 times, at 12% by 10 to 60 times, and at 36% by 13 to 79 times) compared to the survival without sucrose (4.04 ± 0.27 log₁₀ CFU/disk).

In contrast to the effects of sucrose on increasing the survival, addition of NaCl to bacterial solutions decreased the number of bacteria surviving the 24 h of drying. The populations of the 15 strains in paper disks after the 24 h of drying following the addition of bacterial solution with 5% NaCl were 2.80 ± 0.21 log₁₀ CFU/disk, which was approximately 1.5 log₁₀ less than the population for the control with 0.85% NaCl (4.29 ± 0.27 log₁₀ CFU/disk). The presence of 10% and 20% NaCl in the bacterial solutions reduced the populations of all of the strains to less than the detection limit (10^2 CFU/disk).

When cocoa drinks having the same sucrose concentrations (4%, 12%, and 36%) were used for bacterial solutions instead of SPS, the populations of these strains in the disks after the 24 h of drying were 6.01 ± 0.50 , 6.12 ± 0.53 , and 6.24 ± 0.42 log₁₀ CFU/disk, respectively. Compared with the populations of corresponding control strains, the populations of bacteria seeded with cocoa drinks containing sucrose were about 2 log₁₀ units higher than those of the controls. An interesting finding here was that the population of these bacteria suspended in a cocoa drink without sucrose was as high as 5.90 ± 0.43 log₁₀

TABLE 3. Effects of storage temperature on survival of desiccated bacteria in dried paper disks

Storage temp (°C)	Bacterial group	No. of strains surviving/no. tested after storage for (days):			
		1	15	35	70
4	<i>Salmonella</i>	5/5	5/5	5/5	5/5
	STEC O157	5/5	5/5	5/5	5/5
	STEC O26	5/5	5/5	5/5	5/5
25	<i>Salmonella</i>	5/5	5/5	5/5	0/5
	STEC O157	5/5	5/5	3/5	0/5
	STEC O26	5/5	5/5	3/5	0/5
35	<i>Salmonella</i>	5/5	5/5	0/5	0/5
	STEC O157	5/5	5/5	0/5	0/5
	STEC O26	5/5	5/5	0/5	0/5

CFU/disk, which was also 2 log₁₀ units higher than those of the controls of corresponding strains.

(ii) **Survivals of the 15 desiccated strains. (a) Effect of storage temperature on survival.** Unlike the case for storage at 4°C, populations of all of the 15 desiccated strains were below the detection level after 35 days of storage at 35°C and after 70 days at 25°C, except for two strains each of STEC O157 and STEC O26, which became undetectable after 35 days of storage (Table 3).

(b) **Effect of ethanol on survival.** When 15% or less concentrated ethanol was absorbed to a dried paper disk containing one each of the 15 desiccated strains, all of these strains survived 1 min of absorption without their populations being affected. When 40% ethanol was absorbed for 1 min, two strains of salmonellae (one each of *S. enterica* serovars Litchfield and Typhimurium) and three strains of STEC (one of O157 and two of O26) survived with a population range of 2.48 to 3.32 log₁₀ CFU/disk. All of the 15 desiccated strains, however, had undetectable populations (10² CFU/disk) after the absorption of 70% ethanol.

(c) **Effect of exposure to high temperature on survival.** One of the most interesting and noteworthy findings in this study was that all of these desiccated bacteria survived a high-temperature exposure to 70°C for 5 h, showing only an approximately 1 log₁₀ (3.55 ± 0.26 log₁₀ CFU/disk) decrease compared to those stored at 35°C for 5 h (4.45 ± 0.17 log₁₀ CFU/disk). It should be remembered that all of the 5 *Salmonella* strains (3.53 ± 0.40 log₁₀ CFU/disk) and 4 of the 10 STEC strains (2.69 ± 0.21 log₁₀ CFU/disk) survived even an exposure to 80°C for 5 h. Only after exposure to 90°C for 5 h did all of these desiccated bacteria reduce their populations to less than the detection limit (10² CFU/disk).

Survival of the 15 bacteria in dry foods after 24 h of drying at 25°C. The population of each of the 15 strains inoculated in dry foods was determined after 24 h of drying at 25°C and compared with the corresponding population of the strain in the dried paper disk (Table 4). Survival rates of most of the 15 strains inoculated in chocolates (14/15) and roasted peanuts (12/15) were more than 100 times higher than the corresponding rates of those inoculated in the dried paper disks. None of the 15 strains inoculated in acid dry foods (dry apples and dry pickled sour plums, both with pHs of <4), however, survived

TABLE 4. Survival of bacteria in dry foods after drying at 25°C for 24 h

Strain no.	Bacterial group	Population, log ₁₀ (dry foods/dried paper disks) in:				
		Chocolates	Peanuts	Potato chips	Dried squid chips	Dried squid (plain)
1	<i>Salmonella</i> spp.	2.08	2.20	1.90	1.95	0.08
2		2.36	2.45	1.98	1.94	0.18
3		2.18	2.08	NT ^a	NT	NT
4		2.32	2.40	NT	NT	NT
13		2.30	1.96	NT	NT	NT
19	STEC O157	1.89	1.86	1.08	1.36	0.28
20		2.04	2.30	NT	NT	NT
21		2.32	2.62	NT	NT	NT
28		2.40	2.30	NT	NT	NT
31		2.08	2.30	NT	NT	NT
34	STEC O26	2.62	2.15	1.65	1.89	0.18
35		2.49	2.34	NT	NT	NT
36		2.32	2.20	NT	NT	NT
46		2.30	2.04	NT	NT	NT
47		2.18	1.98	NT	NT	NT

^a NT, not tested.

the 24 h of drying at 25°C. A noteworthy finding here was that populations of four selected strains (two each of *Salmonella* spp. [strains 1 and 2] and STEC [strains 19 and 34]) inoculated in the dried squid chips containing sucrose were 23 to 89 times higher than those in the dried paper disks, whereas the populations of these strains in dried plain squid without sucrose were almost equal to those in the dried paper disks.

DISCUSSION

A portion of distributed dried squid chips implicated as the cause of one of the largest diffuse outbreaks of gastroenteritis in Japan, involving more than 1,600 patients, was sent to our laboratory in order to ascertain whether or not those chips distributed or manufactured in Aichi prefecture were also contaminated with the causative bacteria, *Salmonella* serovars Oranienburg and Chester. From those chips, with a_w values of 0.52 to 0.56, we isolated both of these serotypes, with calculated populations of 10³ salmonellae/g. In this study, we utilized 18 strains of salmonellae, including these two strains that we isolated from the dried squid chips, and 35 strains of STEC to investigate the desiccation tolerance of these strains. We found that all of the strains of STEC and nontyphoid *Salmonella* serotypes survived at a level of 10³ to 10⁴ CFU/disk in paper disks following an inoculation with 10⁷ organisms after 24 h of drying at 35°C to become desiccated bacteria. A noteworthy finding here was that 15 selected strains of the desiccated STEC and 4 selected strains of the desiccated salmonellae (including *Salmonella* serovars Oranienburg and Chester isolated from the dried squid chips), survived in refrigerated conditions (4°C) for 22 to 24 months. Certain serotypes of *Salmonella*, such as *S. enterica* serovar Senftenberg 775W, have been reported to have desiccation tolerance (11, 18, 22); however, our study is the first to report desiccation tolerance of salmonellae using as many as 15 different serotypes and storage periods of as long as about 2 years under a fixed low a_w. It should be noted that the STEC strains investigated in this study had almost the same desiccation tolerance as nontyphoid

Salmonella spp., although *E. coli* has not been generally considered to be resistant to desiccation (21). Because of the fact that food-borne outbreaks resulting from dry foods contaminated with STEC O157 have been reported several times since the middle of the 1990s, STEC O157 has been suggested to have the ability to resist dryness (8, 14, 19, 24). In this study, we found that all of the 35 STEC strains (15 each of O157 and O26 and 5 of O111) survived 24 h of drying at 35°C, whereas many of the nonpathogenic *E. coli* (60%; 9/15) did not. These results suggest that the desiccation tolerance characteristic of STEC might be a discriminative property of STEC regardless of serotypes or toxin types. All of the desiccated STEC and *Salmonella* strains in the dried paper disks were extinguished after 35 to 70 days of storage at 25°C and 35°C, in spite of their ability to survive 22 to 24 months of storage at 4°C. In other words, both STEC and salmonellae in dry conditions may die in 1 to 2 months when stored at room temperature or higher but may survive for up to 2 years when stored at cold temperature. This finding suggests that preserving dry foods contaminated with these bacteria in a refrigerator might be more dangerous in terms of food safety (8, 25). We observed that all of the *Salmonella* and STEC strains in the dried paper disks survived at pH 4 to 9 but did not survive at pH 3. This observation was in accordance with the finding that these strains could not survive the 24 h of drying when seeded on dried apples and pickled sour plums with pHs of <4. One of the interesting findings in this study was that sucrose increased the survival rates of salmonellae and STEC strains by more than 10 times, whereas NaCl substantially decreased the rates. This observation was in accordance with the finding that populations of these strains seeded on the dried squid chips containing sucrose were about 10 times higher than those seeded on the plain dried squid without sucrose. Accordingly, dry foods containing sucrose can be considered to be more vulnerable in terms of STEC and salmonella contamination than dry foods containing NaCl. Even though the survival of STEC and salmonellae after the initial 24 h of drying was enhanced by the presence of 36% sucrose by 13 to 79 times compared to the survival without sucrose, the survival of those bacteria inoculated in chocolates containing 30 to 40% sucrose was much more enhanced, by 77 to 420 times. Accordingly, the presence of sucrose alone could not explain the enhancement of survival of these bacterial strains in chocolates. Because we found that the populations of STEC and salmonellae inoculated with a cocoa drink without sucrose were 51 to 370 times higher than those inoculated with SPS, it was suggested that a vegetable fat such as cacao butter, which is an ingredient of cocoa as well as chocolates, was also a contributing factor for the enhancement of survival of these strains in dry foods. Another noteworthy finding in this study was that the desiccated STEC and *Salmonella* strains survived the high-temperature exposure to 70°C for 5 h. We are the first to report that desiccated STEC acquires almost the same degree of heat resistance as *Salmonella*, whose resistance has been reported to increase with decreasing a_w levels (2, 3, 6). These observations clearly indicate that heat sterilization procedures against STEC as well as *Salmonella* in dry foods should be distinctively different from those in non-dried foods, which can be accomplished easily after exposure to 70°C for far less than 5 h.

Although the mechanism(s) of the resistance of STEC and

salmonellae to dryness demonstrated in this study, or that of gram-negative bacteria in general, remains to be elucidated, the "water replacement hypothesis" (7, 20) could be a possible explanation for an underlying mechanism. In this hypothesis, the nonreducing disaccharides sucrose and trehalose are considered to preserve the structure of protein and thus the function of membranes through the inhibition of structural damage by replacing bacterial membranous water in desiccated conditions (4, 7, 17, 20). Accordingly, different resistances to dryness among bacterial strains or the increased survival after drying in the presence of sucrose might be related to abilities of bacteria to accumulate intracellular sucrose and/or trehalose by de novo synthesis and/or translocation (10, 17).

Regarding the mechanism(s) of heat resistance of these STEC strains and salmonellae as "desiccated bacteria," it is reasonable to consider that very low water content of the bacterial cells plays a pivotal role, for the following reasons. Very low water content in the cells can inhibit or diminish the protein denaturation induced by high-temperature heating through vibration of water molecules to break S-S and hydrogen bonds of intracellular proteins (9). Because of the very low water contents in the desiccated STEC strains and salmonellae, the extent and strength of the vibration of water molecules in these bacteria are considered to be limited substantially, thus preventing the bacteria from denaturation of the membrane proteins and preserving their integrity even during exposure to a very high temperature (2).

In conclusion, the data presented here show that the ability of STEC, as well as *Salmonella*, to survive as desiccated bacteria is remarkably enhanced in the presence of sucrose and that the desiccated STEC and *Salmonella* strains have acquired the remarkable characteristics of being able to survive for up to 2 years in refrigerated conditions and of being able to survive 5 h of exposure to 70°C. The findings presented here will give very important and valuable information for risk assessment for dry food safety with regard to STEC and salmonellae in various stages of food supplies from manufacturing to home storage.

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