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Relaying to Decrease the Concentration of Oyster-Associated Pathogens

DAVID W. COOK[1*] and R. D. ELLENDER[2]

Microbiology Section, Gulf Coast Research Laboratory, Ocean Springs, Mississippi 39564, and Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, Mississippi 39406

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ABSTRACT

Oysters experimentally contaminated with indicator bacteria, *Salmonella* and poliovirus were used in relaying studies designed to measure microbial elimination under a variety of environmental conditions. Two factors, level of microorganism in the oyster and temperature of the water, were important in determining the length of time necessary to purge the contaminating organisms. Oysters under physiological stress cleansed at a slower rate than did healthy oysters. Based on the expected level of pathogen contamination in naturally polluted oysters, healthy relaid oysters were capable of cleansing in a 7-d period provided the temperature was above 10°C. These results were verified by following the elimination of indicator bacteria and poliovirus in commercially relaid oysters. Fecal indicator bacteria and enteric pathogenic bacteria were eliminated at similar rates but fecal coliform levels did not correlate with virus elimination. Relaying waters may contain some indicator bacteria and this study suggested that fecal coliforms may not be useful as end-point indicators for this method of oyster purification.

Depuration (*7,9*) and relaying (*3,25,28*) are methods widely recognized for the self purification of contaminated oysters. They rely on the ability of the oyster to eliminate contaminating microorganisms from the digestive tract through normal feeding, digestion and excretion activities. In depuration, man attempts to provide stable environmental conditions (temperature, salinity), and enteric microorganism-free water conducive to the cleansing process. In relaying, man has no control over environmental factors other than time-of-year and site selection, and oysters may be subjected to considerable environmental fluctuation during the purification period.

The filter feeding mechanism of the oyster (*10,11,18*) is the process by which contamination occurs. Bacteria (*13,21,26,27,28*), viruses (*5,6,8,13,22,30*), and other microorganisms as well as foodstuff (*23*) are ingested. The rapidity by which contaminating organisms are excreted depends on the quality of the water being filtered. Salmonellae elimination can occur as rapidly as 3 d (*28*) if optimal conditions exist or be extended to as long as 49 d (*17*). Elimination of enteric viruses by shellfish during depuration (*12*) varies extensively (24 h to 7 d), and depends upon temperature, salinity, and/or the type of depuration system. Indicator bacteria in oysters decrease to a level of <50/100 g within 48 h if temperature and salinity are optimal (*3,21,24*).

Depuration of oysters for commercial purposes is expensive and is practiced on only a limited basis in the United States. Relaying is an economically feasible method of purifying oysters, which is practiced in most Gulf states and will probably expand in the future. Our knowledge about elimination of bacteria and viruses from Gulf oysters has been gained from depuration studies conducted using stable environmental conditions of experimental systems. Data are not available to document the elimination of pathogenic bacteria and viruses from Gulf coast oysters under the naturally fluctuating environmental conditions to which relaid oysters are subjected. Therefore, this investigation was initiated to document the length of time required to reach acceptable microbial levels by this process, to determine if fecal coliforms in oysters are adequate indicators of pathogen elimination and to examine the environmental conditions which maximize the cleaning rate.

MATERIALS AND METHODS

Bacteriological analysis

Fecal coliform concentrations in seawater and oyster samples were analyzed according to standard procedures (*1*); counts were determined using the Most Probable Number (MPN) technique and are expressed as the MPN per 100 g of oyster tissue or 100 ml of water. The size of the shucked oyster sample was approximately 200 g (8-12 oysters, including liquor).

Oysters for salmonellae analysis were shucked and drained for 30 min. Approximately 200 g of oyster meats were

[1]*Inquiries: Gulf Coast Research Laboratory, Ocean Springs, MS 39564.*
[2]*University of Southern Mississippi.*

homogenized with lactose broth to produce a 1 to 10 dilution. A portion of this homogenate was placed in sterile tubes and diluted with lactose broth to achieve subsamples containing 1 to 10^{-5} g of oyster tissue in decmial dilutions. Following a preenrichment period of 24 h, samples were selectively enriched in tetrathionate broth (TET) and selinite cystine broth (SC) + 5% dulcitol. After 24 h at 35°C and after an additional 24 h of incubation at 42°C, each sample was plated on brilliant green agar with and without 0.05% sulfapyridine. Suspect colonies were tested on triple sugar iron agar (TSI) and lysine iron agar (LIA). In studies conducted with known salmonellae, conclusions as to the presence or absence of salmonellae were usually based on the reaction on TSI and LIA media. If reactions were not definitive, additional biochemical tests were done. A sample was considered positive if salmonellae were detected on any of the plating media from either enrichment broth.

Viral analysis

The shells of oysters were scrubbed to remove loose material, rinsed in cold tap water, and disinfected by dipping in 70% ethanol. The oysters were opened, drained of liquor, and the meats transferred to an aluminum screen or stainless steel pan with a perforated bottom for draining (30 min). The meats were packaged in sterile plastic bags (150 g) and frozen at -70°C. Samples were extracted and concentrated as previously described (*6*), and the concentrates assayed immediately or frozen at -70°C for subsequent analysis. Poliovirus quantitation was accomplished by plaque assay in Buffalo green monkey kidney (BGM) cells. Flasks received 0.2 to 0.5 ml inoculum per 25 cm^2. Virus was allowed to adsorb to the cell sheet at 37°C for 1 h on a rocking apparatus (Bellco) at 5 rotations per minute. Plaques were counted daily for 6 d or until no new plaques appeared for two consecutive days. Virus data were expressed as the number of plaque-forming units (pfu) per 100 g of oyster tissue.

Contamination of oysters

Fecal coliform bacteria isolated from estuarine water had an IMViC code of + + − − (*1*). The two strains of salmonellae used most frequently were *Salmonella typhimurium* (American Type Culture Collection, ATCC 13311), and *Salmonellae montevideo* which had been isolated from shrimp. Both of these salmonellae have been isolated from oysters (*2,21*). Poliovirus type 1 (USM isolate 140-44) had been isolated from oysters collected in prohibited oyster growing waters. Virus pools were prepared in BGM cells and monodispersed suspensions titered and maintained at -70°C.

Bacterial and viral preparations were mixed with fecal material for delivery to oyster populations (*20*). Fecal material was collected from a healthy male volunteer and assayed for virus; the stool was negative. The feces were blended with distilled water to yield a 10% suspension, and the preparation frozen at -70°C. Before use, the fecal and viral suspensions were thawed rapidly to room temperature. To 100 ml of the fecal suspension, the desired amounts of virus, salmonellae and/or indicator bacteria were added, and the resulting mixture was shaken for 2 min before addition to the inoculum tank.

Oyster harvesting and preparation

Oysters were collected from two prohibited locations (Graveline Bayou and Biloxi Bay) using a hand dredge and culled to remove spat and excess shell. The oysters were washed with a high pressure water spray to dislodge sediment; only oysters of legal market size (length ≥75 cm, hinge to bill) were used. The oysters (approximately 80) were placed in single layers in baskets (75 × 75 × 7.5 cm) constructed of 2.5 cm square mesh, vinyl-coated, hardware cloth. This quantity of oysters was sufficient to yield two 200-g samples for fecal coliform analysis, two 150-g samples for viral analysis, and one 200-g sample for salmonellae analysis. Baskets of oysters were placed in a tank of bay water at ambient temperature for a few hours to allow the oysters to purge mud from within the shell.

Contamination procedure

Oysters were contaminated by allowing them to feed in water which had been inoculated with the desired microorganisms. The contamination tank (91 × 91 × 91 cm) was constructed of plywood and sealed with fiberglass resin; foamed plastic was glued to the exterior of the tank to retard heat loss. The water was aerated by two large air stones located at opposite corners of the tank. Water in the tank was continually mixed by two submersible pumps located on the bottom in opposite corners of the tank, which discharged the water at the surface near the center of the tank. Mixing time for the approximately 400 L of water in the tank was calculated at <30 min. The water temperature was elevated above ambient when necessary by a submersible heater.

Oysters that were harvested during times of the year when water and air temperatures were equal to or greater than 20°C were contaminated under ambient temperature conditions. However, during the winter, it was necessary to acclimate the oysters to a temperature of about 20°C before contamination. The warmer temperature assured that the oysters would actively take up the microbial inoculum. Acclimation was accomplished by placing the purged oysters in the tank filled with water at ambient temperature, and slowly raising the temperature of the water to 20°C. This was usually accomplished over a 6-h period.

Baskets of acclimated oysters were removed from the tank; the inoculum was added and allowed to disperse in the water for 15 min. The oysters were then returned to the tank and allowed to accumulate the microorganisms over a 14- to 16-h period, after which the baskets were removed and the oysters washed with a chlorine spray to kill pathogens on the shell.

Relaying procedure and sites

Immediately after contamination, the baskets of oysters were transported to the relaying site and lowered to the bottom. The baskets were tethered to a piling used to mark the relaying site. The baskets were not disturbed between relaying and harvesting. On each sampling day, one basket of oysters was retrieved and returned to the laboratory for analysis. Figure 1 shows the location of the three experimental oyster relaying sites and Table 1 lists the temperature and salinity in the area during each study. All sites were in conditionally approved waters. The Bellefontaine site was adjacent to an oyster lease which had been used for relaying. During periods of heavy river flow, the water quality in that area deteriorated. The Deer Island site was less subject to water quality deterioration during heavy rainfall periods, but had high salinities during periods of low rainfall. The Ship Island site was used for the high salinity studies.

Studies with commercially relaid shellfish

The commercial relaying projects followed in this study were carried out by the Bureau of Marine Resources, Mississippi De-

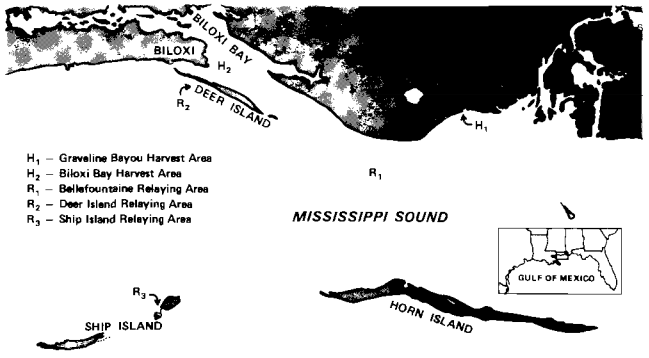

Figure 1. *Experimental oyster relaying sites.*

TABLE 1. *Location, temperature and salinity of relaying sites.*

Relaying study number	Relaying area (see map)	Temperature (°C)	Salinity (ppt)
1A	R_1	17.7±2.3[a]	21.3±4.5
1B	R_1	10.7±2.6	20.8±4.5
1C	R_1	23.2±2.1	17.7±6.4
2B	R_1	29.1±0.9	21.4±5.5
2C	R_2	30.4±1.5	24.0±1.4
3A	R_1	13.7±2.8	21.8±5.1
3B	R_1	17.4±1.1	13.6±5.9
7A	R_2	10.9±3.0	23.7±1.4
7B	R_2	15.0±1.1	24.8±1.1
7C	R_2	10.4±1.9	27.7±3.8
7D	R_2	15.1±2.3	21.0±3.6
8A	R_3	29.3±0.7	27.6±1.3
8B	R_3	29.2±0.8	26.6±0.9

[a]Mean ± standard deviation.

partment of Wildlife Conservation. Under this program, oysters were harvested by a state-operated dredge boat, transported to the relaying site, and shoveled overboard. Samples were collected from the dredge boat before unloading and following an appropriate relaying period.

Water sampling

When oysters were harvested, two surface and two bottom water samples were collected at the sampling site for bacteriological analysis and environmental parameter measurements. Surface water samples were collected in a sterile bottle by the grab technique. A bacteriological water sampler (CM^2, Mountain View, CA) was used to collect bottom samples. Samples were placed on ice and returned to the laboratory. Salinity was determined with an American Optical Refractometer and recorded in parts per thousand (ppt). Temperatures, were measured in situ with a mercury-in-glass thermometer, and recorded to the nearest 0.5°Celsius (C).

RESULTS

Relaying studies with inoculated oysters

Table 2 shows the decrease in numbers of fecal coliforms in groups of oysters held in relaying waters where the fecal coliform levels remained below 14 per 100 ml during the study period. With the exception of oysters inoculated with high levels of fecal coliforms (Study 1A) and the oysters held near 10 C (Study 7C), the fecal coliform levels were reduced >99.9% within 5 d.

S. typhimurium levels in oysters were reduced at a rate equal to the fecal coliforms and were not detected in samples collected 4 to 11 d following relaying (Table 3). *S. montevideo* levels were reduced rapidly following relaying, but, as shown in Table 4, remained in the oysters at low but detectable levels for greater than 15 d.

Eight poliovirus elimination studies were conducted (Table 5). In four of these studies, infectious virus was not detected in the oysters 7 d after relaying; and in five of the studies, tests were negative for viruses by the fifteenth day. The three groups of oysters which failed to eliminate the poliovirus had been inoculated with heavy doses of virus (13,000 to 35,000 pfu/100 g). However, one group of oysters (8A), which had been inoculated with 22,000 pfu/100 g, eliminated them in less than 5 d.

The temperature to which the oysters were exposed following relaying appeared to affect the virus elimination rate. Oysters which had accumulated large numbers of poliovirus and were held at temperatures less than 20°C failed to eliminate within 2 weeks, whereas those held at high temperatures (>25°C) eliminated in less than 1 week. Oysters inoculated with large numbers of virus particles and held at low temperatures (<14°C) retained virus for >32 d.

Studies with commercial relaying

Microbiological studies were conducted in connection with two lots of oysters relaid as part of a commercial operation. In the first of these, viruses were detected in only 1 of 3 150-g samples taken at the time of harvest. Salmonellae were not detected in a single 200-g sample. Table 6 shows that the fecal coliforms and viruses were rapidly eliminated from the oysters.

Viruses were found in two 150-g samples taken from the second lot of commercially relaid oysters at the time of harvest. The levels were 6.6 and 5.5 pfu/100 g. No salmonellae were found in a single 200-g sample of the oysters. Samples taken 1 and 2 d after relaying were free of viruses.

DISCUSSION

These studies have confirmed that relaying is an effective means of decreasing the level of indicator bacteria and enteric pathogens in contaminated oysters. As one would expect, the rate at which the microorganisms are reduced is slower in relaid oysters than in depurated oysters and this can be attributed to the fluctuation in temperature and salinity, deviation from ideal temperature and salinities, and mechanical disturbance of the animals by currents and animals.

In general, oysters were found to reduce *S. typhimurium* to a low level within <5 d after relaying, and to a non-detectable level (<1 per 200 g) within 5 to 9 d under a wide variety of environmental conditions. Similar results have been obtained by other researchers (*21,28*). *S. montevideo* responded with an initial reduc-

TABLE 2. *Elimination of fecal coliforms from experimentally contaminated oysters following relaying to the study area. In each study the average fecal coliform levels in the bottom water at the relaying site on each sampling day were <14/100 ml.*

Time	Relaying study number						
	1A	1C	2B	2C	7C	8A	8B
Day 0	13,000,000[a]	24,000	6,400	5,200	20,000	28,000	6,400
1	180,000	200,000	3,300	450	10,000	440	360
2	310,000						
3			65	190	2,000	<49	<20
4		20					
5	15,000		<20	<40		<20	
6		<20					<20
7					1,200		
9		<20	20	<20			
10	10,000					<20	
12					110		<20
13			<20				
15		<20		<40			
17	2,800						

[a]Fecal coliform MPN per 100 g of oyster tissue.

TABLE 3. *Elimination of* Salmonella typhimurium *from experimentally-contaminated oysters following relaying to study areas.*

Time	Relaying study number				
	1A	1B	3A	3B	7A
Day 0	10^{3}[a]	$\geq 10^{3}$	10^{4}	$\geq 10^{4}$	$\geq 10^{5}$
1	10^{2}	10	10^{4}	$\geq 10^{3}$	10^{4}
2	ND[b]		$<10^{3}$		
4		ND		ND	
5	<1				ND
6			<1	1	
8					ND
10	ND	ND			
11			ND		

[a]Estimated number of salmonellae per gram of oyster tissue.
[b]Salmonellae not detected in 200-g sample.

tion to levels of ≤1 cell per gram in 3 to 8 d after relaying, but persisted in oysters at a detectable level (>1 to <200 cells/200 g) for as long as 28 d. Failure of the oysters to eliminate this species is not understood, but may signal that low levels of other species of salmonellae could persist for lengthy periods in oysters, or that failure to detect other salmonellae species is related to the techniques of recovery.

When considering the failure of oysters to eliminate all salmonellae, several facts must be reviewed to place the public health aspects in perspective. The oysters used in these studies were heavily contaminated with salmonellae. There is a notable lack of information in the literature on the level of salmonellae in oysters from sewage-contaminated waters, but the available evidence indicates that the number of salmonellae in the oysters would be expected to be low except under extreme pollution conditions. In most studies, the oysters eliminated greater than 99.9% of the salmonellae within 7 d after relaying. Also, the available literature (*15,19*) indicates that a massive dose of salmonellae must be ingested for illness to ensue. Further, Son and Fleet (*28*) have shown that salmonellae do not multiply in oysters stored in the shell at room temperature. A rational consideration of this information leads to the conclusion that relaying is an effective means of eliminating the public health problem caused by salmonellae in oysters.

In several studies in which the oysters were exposed to, and accumulated, large numbers of virus particles, they failed to eliminate the viruses after relaying. The concentration of viruses which the oysters accumulated exceeded those which oysters in nature accumulate by several thousand fold. Although we have no direct supporting evidence, we offer the following speculative theory as to why these oysters failed to eliminate.

Virus particles readily adsorb to suspended solids in the water column. In sewage-polluted estuarine waters, viruses taken in by oysters are probably adsorbed to and remain attached to particulate matter as they pass through the oyster digestive system. In the experimental relaying studies, we attempted to adsorb cell-culture grown virus to suspended particles of human feces. When low numbers of viral particles were used, all may have become particle-associated, a factor which hastened their movement through the oyster's digestive system. In instances when large numbers of viruses were added, some of the viruses may not have become particle-associated. Those "free" viruses may have become entrapped in mucus, and entered the oyster's digestive system. Because of their small size, they may have been carried into areas of the digestive diverticulum from which they were more difficult to dislodge, or phagocytised and carried by the circulatory system into other tissues. This theory is in agreement with observations of Metcalf et al. (*20*). Further, Hamblet et al. (*14*) offer data for indirect support of this theory by showing that virus which accumulates in turbid water is rapidly eliminated in depuration studies.

TABLE 4. *Elimination of* Salmonella montevideo *from experimentally contaminated oysters following relaying to study areas.*

Time	Relaying study number							
	1C	2B	2C	7B	7C	7D	8A	8B
Day 0	$\geq 10^4$[a]	$\geq 10^4$	$\geq 10^4$	≥ 10	$\geq 10^3$	10	$\geq 10^4$	10^3
1	$\geq 10^2$	10^2		≥ 10	10^2	10	$\geq 10^2$	$\geq 10^2$
3	1	$\geq 10^2$	10	1		10	10	10
4					$\geq 10^2$			
5	10	$\geq 10^2$	1	<1			10	
6						<1		<1
7					$\geq 10^2$			
8				<1				
9	1	1	10			<1		
10							1	
12					<1			ND
13		<1		<1				
15	ND[b]		1				1	
16						<1		
20		<1						
22					<1		ND	
24						ND		
25			ND					
28					<1			

[a]Estimated number of salmonellae per gr of oyster tissue.
[b]Salmonellae not detected in 200-g sample.

TABLE 5. *Elimination of poliovirus from experimentally contaminated oysters following relaying to study area.*

Time	Relaying study number							
	1A	1B	1C	7A	7B	7C	8A	8B
Day 0	35,000[a]	34,000	27.6	13,000	16	71	22,000	43,000
1	160	3,500		3,000	1.3	19	3,200	28
2	780		20.4					
3					0.8		2.5	ND
4		120	5.3			0.8		
5	590			19	ND		ND	
6			1.3					
7						ND		ND
8				10	ND			
9			1.3					
10	23	8					ND	
12						ND		
14				10				
15		12	ND[b]					
17	50							
28				4				
30			ND					
31		4						
45				ND				

[a]Plaque forming units per 100 g of oyster tissue.
[b]Virus not detected.

TABLE 6. *Changes in the microbiological quality of oysters relaid from a polluted to a conditionally approved area.*

Source	Water			Oysters		
	Salinity (ppt)	Temperature (°C)	Fecal Coliforms (MPN/100 ml)	Fecal Coliforms (MPN/100 g)	Salmonellae (No./100 g)	Viruses (PFU/100 g)
Harvest Area	15	30	43	380	ND[a]	2.4
Relaying Area						
Day 0	25	30	<2	--	--	--
Day 2	25	--	4.9	88	ND	ND
Day 5	22	--	<2	<20	ND	ND

[a]ND - None detected.

The maximum number of virus pfu found in oysters harvested from reefs closed to shellfish harvesting has been about 200/100 g (*13*). Our data indicate that under acceptable environmental conditions, oysters would be expected to eliminate those enteric viruses present at levels of 200 pfu/100 g or below within a 7-d period.

Fecal coliform elimination proceeded rapidly following relaying with greater than 99.9% reduction within 3 d. However, the final level to which the fecal coliforms were reduced reflects the level of fecal coliforms in the water at the relaying site.

In field studies it is difficult to determine which environmental factors produce specific effects. However, from the data presented, certain inferences can be made concerning the effects of temperature and salinity.

In general, temperature had an identifiable effect on the rate at which enteric bacteria and viruses were eliminated from oysters. Fecal coliforms were eliminated at a faster rate at higher water temperatures as noted when comparing studies 7C and 8A (Table 2). Similar trends were noted with poliovirus when studies 7A and 8A (Table 5) were compared. There was little identifiable effect of temperature on salmonellae elimination. Under all temperature conditions salmonellae reached low levels quickly.

In estuarine waters of the Gulf Coast which are of sufficient depth to permit relaying, the water temperatures occasionally rise to, but seldom exceed, 31°C. Temperatures at this level do not have an adverse effect on the oyster's ability to eliminate enteric microorganisms. In those same waters, temperatures do fall below 10°C and at that temperature, oyster activity, and enteric microorganism elimination is slowed.

The salinity of the water into which oysters are relaid appears to have little effect on their ability to eliminate enteric microbes, provided the salinity was sufficient to maintain metabolic functions. High salinities approaching 30 ppt did not appear to adversely affect the cleansing of the oysters. Many of the low salinity areas (<15 ppt) in Mississippi failed to meet bacteriological standards (*4*), and for this reason could not be used for oyster relaying even though cleansing is technically feasible.

Implications of these studies are that relaying is an effective means of elminiating enteric microorganisms from Gulf Coast oysters. However, the length of time required to remove enteric microorganisms from experimentally contaminated oysters by relaying was greater than what has been required in depuration systems.

The concentration of enteric microorganisms in the oysters and the temperature of the water at the relaying site were the most important factors in determining the time required for the oysters to decontaminate. Oysters under severe physiological stress failed to eliminate as quickly as healthy oysters (data not presented).

Available data indicate that the number of enteric microorganisms normally found in Gulf Coast oysters could be eliminated by the oysters in less than 7 d following relaying. However, since no data are available on the elimination of hepatitis virus from oysters, it is suggested that the minimum relaying time be set at 14 d, twice that which is adequate for other enteric pathogens. Further, since the water temperature in relaying areas occasionally drops below 10°C, it is suggested that the relaying period for Gulf Coast oysters be extended by 1 d for each day that the bottom water temperature remains below 10°C.

Fecal coliforms have proven useful as bacterial indicators of pollution in shellfish growing waters and of shellfish contaminated during processing. Fecal coliforms are also being considered as end-point indicators for relaying and depuration. Fecal coliforms could adequately serve as end-point indicators only if: (*a*) there is a direct relationship between levels of fecal coliforms and enteric pathogens in oysters, (*b*) enteric pathogens are eliminated at a rate equal to or faster than fecal coliforms, and (*c*) waters in relaying areas are free of fecal coliforms.

Little correlation has been observed between the level of fecal coliforms and viruses in oysters (*5,6,29*). This study has shown that indicator bacteria and enteric pathogens are eliminated at similar rates, but in some instances, viruses were detected after the fecal coliform level had reached <50 per 100 g (study 1C, Table 2 and 5). Waters into which oysters are relaid are permitted to contain some indicator bacteria (*16*). Considering the ability of the oysters to concentrate bacteria from the water, the level of fecal coliforms in oysters may remain high even though pathogens are being eliminated. It appears, therefore, that fecal coliforms would not be useful as end-point indicators in relaying.

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